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THE BIOLOGICAL BULLETIN

PUBLISHED BY
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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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S. Meryl Rose, University of Illinois
Mary Sears, Woods Hole Oceanographic Institution
Albert Tyler, California Institute of Technology

TO SERVE UNTIL 1961

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G. Failla, Columbia University, College of Physicians & Surgeons
Eric Ball, Harvard University Medical School
R. T. Kempton, Vassar College
L. H. Kleinholz, Reed College
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TO SERVE UNTIL 1960

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

Gentlemen:

I submit herewith the report of the seventy-first session of the Marine Biological Laboratory.

1. *Attendance*

Attendance has continued at a fairly constant level through the past several years as shown in the Tabular View of Attendance. Only minor differences in attendance occur from year to year since the laboratory space is fully occupied and the classes are at capacity. Recently there have been qualified investigators with problems appropriate to the Laboratory's research materials and facilities who could not be accommodated. This has posed a difficult problem for the Research Space Committee in the assignment of space. It frequently has been difficult for the Committee to avoid rather arbitrary decisions. It is anticipated that this problem will be eased with the completion of the new research laboratory in the spring of 1960.

2. *Crane Building*

Renovation of the Crane Building carried out under a grant by the National Science Foundation was completed on schedule, permitting occupancy on June 1, 1958. Space utilization has been greatly improved by the rearrangement of facilities within the laboratories. Also the addition of new utilities has resulted in a real improvement in scientific working conditions.

3. *New Laboratory Building*

During the past winter the Building Committee has had several meetings with the architects for the new building (Sheply, Bulfinch, Richardson and Abbott) and, with the plans already well developed, construction will start in May, 1959, the building to be completed and ready for summer occupancy in 1960. The building will have a ground level basement and three floors. Half of the basement will be given over to laboratories, the other half to utilities and services. There will be radiobiological service laboratories on the top floor. A caesium ^{137} radiation unit, a new departure in radiobiological technique, is being planned and will be constructed under the direction of Dr. G. Failla and installed in one of the radiobiological service laboratories.

Attached to the new building will be a lecture room seating 150. The experience gained in renovating the Crane Wing is being used to advantage by the Building Committee.

4. Devil's Lane Housing Project

The attendance of younger investigators at the Laboratory, particularly those with families, has frequently been limited by the cost of housing rental. The Laboratory applied to the National Science Foundation and received a grant for housing construction on the Laboratory's Devil's Lane property. Twenty-four cottages are being erected and will be ready for 1959 summer occupancy. Through the generosity of the Grass Foundation another cottage is being added to those above. Through the efforts of Mr. Smith, General Manager, the Laboratory has a tax exemption on this new housing project which will permit modest rental changes.

The recently completed and the projected construction at the Laboratory has been made possible by the grants listed below:

National Science Foundation—Crane Wing	$415,000
National Science Foundation—New laboratory and housing	544,250
National Institutes of Health—New laboratory	369,250
Rockefeller Foundation—New laboratory	738,500
Grass Foundation—Housing	10,000

5. Grants, Contracts and Contributions

The total income to the laboratory from these sources of support amounted to $184,445.23 in 1958. This represents 34.3% of the total income and is made up of the following accounts:

American Cancer Society—RC4A(+)—Studies in Radiobiology	$ 6,600.00
AEC—1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	10,775.00
NIH—4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms	40,000.00
NIH—C3813 (A) Bio-Equipment	1,600.00
NSF—5143—Training Program in Nerve Muscle Physiology	51,593.00
ONR—1497—Studies in Marine Biology	15,000.00
ONR—09701—Studies on Isolated Nerve Fibers	6,712.23
ONR—09702—Studies in Ecology	6,936.00
M.B.L. Associates	3,150.00
Abbott Laboratories	1,000.00
American Philosophical Society	2,500.00
Carter Products, Inc.	1,000.00
Ciba Pharmaceutical Products, Inc.	1,000.00
Josephine B. Crane Foundation	2,000.00
Eli Lilly and Company	5,000.00
Hoffman-LaRoche, Inc.	1,000.00
Merck Company Foundation	1,000.00
Pfizer Foundation, Inc.	1,000.00

Rockefeller Foundation	20,000.00
Schering Foundation, Inc.	1,000.00
Smith, Kline, and French Foundation	3,000.00
The Upjohn Company	1,000.00
Wyeth Laboratories	1,000.00
Miscellaneous Individuals	579.00
	$184,445.23

It is gratifying that such a diverse group of agencies, foundations, companies and individuals as listed above are interested in the support and development of the Laboratory and its research programs.

6. *Courses*

The course in Physiology will be modified and operate in 1959 as a Physiology Training Program under the direction of Dr. William D. McElroy with financial support from the National Institutes of Health. The additional support will permit well-qualified doctoral and post-doctoral students to participate in the program who otherwise might not be able to do so. Selected students will continue on in the program doing research through the last half of the summer following the completion of the earlier formal part of the program.

With the loss of certain dormitory facilities, the Old Rockefeller Building is being converted into a dormitory for some of the summer service personnel. The course in Marine Ecology which formerly occupied this building is being moved into the Old Main Building.

7. *Boats*

Two new collecting boats were purchased and were received by the Laboratory in May, 1958. These are 24-foot open-cockpit boats of rugged construction and have proved very sea-worthy. One is used for the daily trips to the fish traps and, together with the second boat, for inshore collecting. They replace two old boats, the *Sagitta* and *Tern.* A diesel engine was installed in the *Arbacia* resulting in real economies in operation.

8. *Survey*

During the summer of 1958 the firm of Shurcliff and Merrill, Landscape Architects and Town Planners, was retained jointly by the Marine Biological Laboratory and the Woods Hole Oceanographic Institution to survey Woods Hole, including the physical lay-out of each institution on its own campus and also problems of the institutions as they relate to the community. The resulting report emphasized the present wasteful utilization of the six acres of land which make up the central campus of the M.B.L. The six frame houses, formerly private dwellings, which are used as dormitories occupy an excessive amount of land for the number of people they accommodate. They are old buildings, ill adapted for dormitory purposes and should be replaced. The old Mess Hall, adapted to self-service, could advantageously be replaced by a new building in combination with dormitory facilities. Consolidation of these facilities will open up areas urgently needed for parking. With the development of additional training programs, there will develop a need for training facilities space. The Old Lecture Hall can readily be converted to such temporary use but it will not be entirely satisfactory for a program involving the new techniques required for a modern training program. Only a new building will adequately serve this purpose.

Respectfully submitted,
Philip B. Armstrong,
Director

MEMORIAL

Allen R. Memhard

by

Donald P. Costello

Allen R. Memhard had a lifelong interest in biology, but, by reason of training for the Bar and practicing international and corporation law, was able to indulge this interest only in his later life.

Mr. Memhard was born in Chicago, and was graduated from the New York Law School in 1915. Soon after his graduation, he began an independent law practice in New York City. This was interrupted briefly while he served in U. S. Army Intelligence during World War I, after which he returned to his own practice. Later he became a member of the law firm of O'Brien, Boardman, Fox, Memhard and Early. In 1933 he was a delegate to a conference on international law at The Hague. From 1939 to 1950 he was counsel for the Geological Society of America. He was also very active in town affairs in his home community of Greenwich, Connecticut. Here he and Mrs. Memhard raised their fine family of two daughters and three sons.

In 1941, to learn more about the field that had interested him since childhood, he enrolled in a course in biology at New York University, where he came into contact with the late Professor Robert Chambers. By this time, Mr. Memhard was in a position to take time from his professional work to pursue his interest in marine biology, and Dr. Chambers was so impressed with his sincerity and abilities that he encouraged him to apply for space at the Marine Biological Laboratory. During the summer of 1942, Mr. Memhard first took the Embryology Course, which was under the direction of Professor Viktor Hamburger, and then was engaged in research during the remainder of the summer. He continued his research work in marine embryology during the summers of 1943, 1944, 1945, and part of 1947. He was elected a member of the Corporation in 1945.

In 1946, Mr. Memhard established a scholarship fund at the Marine Biological Laboratory, the income of which is awarded to qualified students who complete the course in embryology and wish to return or stay on for further work.

Mr. Memhard was a charming gentleman, a diligent scholar, and an interested observer of marine embryological phenomena. He was a fine example of that group of enlightened laymen who deserve the title, "Friend of the Marine Biological Laboratory."

1. The Staff, 1958

Philip B. Armstrong, Director, State University of New York, School of Medicine. Syracuse

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Morrison Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Grover C. Stephens, Assistant Professor of Zoology, University of Minnesota; in charge of course.
John B. Buck, Senior Biologist, National Institutes of Health
Demorest Davenport, Associate Professor of Biology, Santa Barbara College
Peter W. Frank, Associate Professor of Biology, University of Oregon
Clark P. Read, Associate Professor, School of Hygiene and Public Health, Johns Hopkins University
Morris Rockstein, Associate Professor of Physiology, New York University College of Medicine
Howard A. Schneiderman, Associate Professor of Zoology, Cornell University
Milton Fingerman, Assistant Professor of Zoology, Tulane University

III. Laboratory Assistants

Frank E. Friedl, University of Minnesota
Irwin W. Sherman, Northwestern University

EMBRYOLOGY

I. Instructors

Mac V. Edds, Jr., Professor of Biology, Brown University; in charge of course
Philip Grant, Assistant Professor of Pathobiology, Johns Hopkins University
John W. Saunders, Jr., Professor of Zoology, Marquette University
Nelson T. Spratt, Jr., Professor of Zoology, University of Minnesota
Maurice Sussman, Associate Professor of Biological Sciences, Northwestern University
Lionel Rebhun, Assistant Professor of Anatomy, University of Illinois

II. Laboratory Assistants

Chandler M. Fulton, Rockefeller Institute for Medical Research
David S. Love, University of Colorado

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory

II. Instructors

W. D. McElroy, Professor of Biology, Johns Hopkins University; in charge of course
Francis D. Carlson, Assistant Professor of Biophysics, Johns Hopkins University
Bernard D. Davis, Professor of Bacteriology, Harvard Medical School
Donald Griffin, Professor of Zoology, Harvard University
Howard Schachman, Virus Laboratory, University of California
Albert W. Frenkel, University of Minnesota

III. Laboratory Assistant

Louis Otero, University of Puerto Rico, Rio Piedras

BOTANY

I. Consultant

Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Botany, University of Texas; in charge of course
John M. Kingsbury, Assistant Professor of Botany, Cornell University
Richard C. Starr, Associate Professor of Botany, Indiana University

III. Lecturer

Ruth Patrick, Curator of Limnology, Academy of Natural Sciences of Philadelphia

IV. Laboratory Assistants

Temd R. Deason, University of Texas
Harry W. Bischoff, Texas Lutheran College

ECOLOGY

I. Consultants

Paul Galtsoff, U. S. Fish and Wildlife Service, Woods Hole
Alfred C. Redfield, Woods Hole Oceanographic Institution
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
Charles E. Jenner, University of North Carolina
Howard L. Sanders, Woods Hole Oceanographic Institution

II. Instructors

Howard T. Odum, Director, Institute of Marine Science, University of Texas
John H. Ryther, Marine Biologist, Woods Hole Oceanographic Institution
Albert J. Bernatowicz, Chairman, Department of Botany, University of Hawaii

III. Laboratory Assistant

Cameron E. Gifford, Harvard University

THE LABORATORY STAFF, 1958

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian

Carl O. Schweidenback, Manager, Supply Department

Robert Kahler, Superintendent, Buildings and Grounds

Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Irvine L. Broadbent, Office Manager

Mrs. Lila S. Myers
Mrs. Vivien R. Brown
Mrs. Virginia M. Morehouse
Mrs. Emily B. Merrill
Mrs. Vivian I. Manson
Mrs. Shirley Elder

LIBRARY

Mary Rohan
Mrs. M. Verna Hanks
Mrs. Naomi Botelho
Albert K. Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Eldon P. Allen
Edmond Botelho
Arthur D. Callahan
Robert Gunning
Walter J. Jaskun
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Alan G. Lunn
Alton J. Pierce
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
John P. Harlow
Seaver R. Harlow
Ludie A. Johnson

SUPPLY DEPARTMENT

Donald P. Burnham
Milton B. Gray
Geoffrey J. Lehy
Robert O. Lehy
Mrs. Mildred Mixson
Robert M. Perry
Bruno Trapasso
Jared L. Vincent
Samuel W. Vincent
Hallett S. Wagstaff

2. Investigators, Lalor and Lillie Fellows, and Students

Independent Investigators, 1958

Afzelius, Bjorn, Assistant Professor of Biophysics, Johns Hopkins University
Allen, M. Jean, Associate Professor of Biology, Wilson College
Allen, Robert D., Assistant Professor of Biology, Princeton University
Armstrong, Philip B., Professor and Chairman, Department of Anatomy, State University of New York, College of Medicine at Syracuse
Arnold, William, Principal Biologist, Division of Biology, Oak Ridge National Laboratory
Baird, Spencer, Associate Researcher, Institute for Muscle Research, Marine Biological Laboratory

Bang, Frederik B., Professor of Pathobiology, Johns Hopkins University School of Medicine
Barth, Lester G., Professor of Zoology, Columbia University
Battley, Edwin H., Instructor in Biochemistry, The Medical Center, Jersey City
Benesch, Reinhold, Investigator, Marine Biological Laboratory
Bennett, Michael V. L., Research Associate, Columbia University, College of Physicians & Surgeons
Berman, Mones, Assistant Physicist, Sloan Kettering Institute
Bernatowicz, Albert J., Chairman, Department of Botany, University of Hawaii
Bettelheim, Frederick A., Assistant Professor in Chemistry, Adelphi College
Bishop, David W., Staff Member, Department of Embryology, Carnegie Inst. of Washington
Bold, Harold C., Professor of Botany, University of Texas
Brown, Frank A., Jr., Morrison Professor of Biology, Northwestern University
Bryant, Shirley H., Assistant Professor of Pharmacology, University of Cincinnati
Buck, John B., Physiologist, National Institutes of Health
Burbanck, W. D., Professor of Biology, Emory University
Carlson, Francis D., Associate Professor of Biophysics, Johns Hopkins University
Case, James F., Assistant Professor of Zoology, State University of Iowa
Del Castillo, Jose, Head, Section of Clinical Neurophysiology, National Institutes of Health
Chaet, Alfred B., Assistant Professor of Physiology, Boston University School of Medicine
Chang, Joseph J., Visiting Scientist, National Institutes of Health
Chase, Aurin M., Associate Professor of Biology, Princeton University
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Child, Frank M., Instructor in Zoology, The University of Chicago
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School
Claudatus, John C., Head, Department of Biochemistry, The Cancer Institute at Miami
Clement, Anthony C., Professor of Biology, Emory University
Clowes, G. H. A., Research Director *Emeritus,* Lilly Research Laboratories
Cole, Kenneth S., Chief, Laboratory of Biophysics, National Institutes of Health
Collier, Jack R., Assistant Professor of Zoology, Louisiana State University
Colwin, Arthur L., Associate Professor, Queens College
Colwin, Laura Hunter, Lecturer, Queens College
Cooperstein, Sherwin J., Associate Professor of Anatomy, Western Reserve University, School of Medicine
Costello, Donald P., Kenan Professor of Zoology, University of North Carolina
Cowgill, Robert W., Assistant Professor of Biochemistry, University of Colorado, School of Medicine
Crane, Robert K., Associate Professor of Biological Chemistry, Washington University Medical School
Crowell, Sears, Associate Professor of Zoology, Indiana University
Csapo, Arpad, Associate Professor, Rockefeller Institute for Medical Research
Davenport, Demorest, Associate Professor in Zoology, University of California, Santa Barbara College
Davis, Bernard D., Professor of Bacteriology, Harvard Medical School
Eccles, Rosamond, Research Fellow, Australian National University
Edds, Mac V., Jr., Professor of Biology, Brown University
Failla, G., Professor, Columbia University
Fankuchen, Isidor, Professor and Head, Division of Applied Physics, Polytechnic Institute
Fawcett, Don W., Professor of Anatomy, Cornell University Medical College
Fingerman, Milton, Assistant Professor of Zoology, Newcomb College, Tulane University
Fishman, Louis, Research Associate, New York University College of Dentistry
Frank, Peter W., Associate Professor of Biology, University of Oregon
Frenkel, Albert W., Professor of Botany, University of Minnesota
Friedl, Frank E., Teaching Assistant, University of Minnesota
Galtsoff, Paul S., Director, U. S. Fish & Wildlife Service, Department of the Interior
Goldberg, Irving H., Post-Doctoral Student, Rockefeller Institute for Medical Research
Goto, Masayosi, Investigator, Rockefeller Institute for Medical Research
Grant, Philip, Assistant Professor of Pathobiology, Johns Hopkins University
Green, James W., Associate Professor of Physiology, Rutgers University

GREIF, ROGER L., Associate Professor of Physiology, Cornell University Medical College
GRIFFIN, DONALD R., Professor of Zoology, Harvard University
GRODZINSKI, F., Professor of Comparative Anatomy, University of Krakow, Poland
GROSCH, DANIEL S., Professor of Genetics, North Carolina State College
GROSS, PAUL R., Associate Professor of Biology, New York University
GRUNDFEST, HARRY, Associate Professor of Neurology, Columbia University, College of Physicians and Surgeons
GUTTMAN, RITA, Assistant Professor of Biology, Brooklyn College
HAGINS, WILLIAM A., Research Medical Officer, Naval Medical Research Institute
HARDING, CLIFFORD V., Visiting Assistant Professor of Zoology, University of Pennsylvania
HARVEY, E. NEWTON, Professor of Biology *emeritus,* Princeton University
HARVEY, ETHEL BROWNE, Investigator in Biological Research, Princeton University
HAYASHI, TERU, Professor of Zoology, Columbia University
HEGYELI, ANDREW, Associate Researcher, Institute for Muscle Research, Marine Biological Laboratory
HEILBRUNN, L. V., Professor of General Physiology, University of Pennsylvania
HENLEY, CATHERINE, Research Associate, University of North Carolina
HERVEY, JOHN P., Senior Electronic Engineer, Rockefeller Institute for Medical Research
HIATT, HOWARD, Associate in Medicine, Harvard Medical School
HICKSON, ANNA KELTCH, Independent Investigator, Lilly Research Laboratories
HOLZ, GEORGE G., JR., Associate Professor of Zoology, Syracuse University
HUGHES, GEORGE M., University Lecturer, University of Cambridge, England
HUSSEY, KATHLEEN L., Assistant Professor of Parasitology, Columbia University
HYDE, BEAL B., Assistant Professor of Plant Sciences, University of Oklahoma
ISENBERG, IRVIN, Associate Researcher, Institute for Muscle Research, Marine Biological Laboratory
ITO, SUSUMU, Instructor in Anatomy, Cornell University Medical College
JACKSON, SYLVIA FITTON, Assistant Professor, Kings College, London
JENNER, CHARLES E., Associate Professor of Zoology, University of North Carolina
JOHNSON, FRANK H., Professor of Biology, Princeton University
KAJI, AKIRA, Fellow in Ophthalmology, Johns Hopkins University
KANE, ROBERT E., Postdoctoral Research Fellow, Johns Hopkins University
KEMPTON, RUDOLF T., Professor of Zoology, Vassar College
KENNEDY, DONALD, Assistant Professor of Zoology, Syracuse University
KINGSBURY, JOHN M., Assistant Professor of Botany, Cornell University
KLEINHOLZ, L. H., Professor of Biology, Reed College
KLOTZ, IRVING M., Professor of Chemistry, Northwestern University
KLUSS, BYRON C., Instructor, Albion College
KÖHLER, KURT, Research Associate, Florida State University
KRAMER, SOL, Special Research Fellow, Marine Biological Laboratory
KRANE, STEPHEN M., Instructor in Medicine, Massachusetts General Hospital
KUFFLER, STEPHEN W., Professor of Ophthalmic Physiology, Johns Hopkins University
KURY, LIVIA REV, Institute for Muscle Research, Marine Biological Laboratory
LANSING, ALBERT I., Professor of Anatomy, University of Pittsburgh
LASH, JAMES W., Instructor in Anatomy, University of Pennsylvania
LENHOFF, HOWARD M., Investigator in Biochemistry, Carnegie Institution
LEVY, MILTON, Professor of Biochemistry, New York University College of Dentistry
LEWIN, RALPH A., Independent Investigator, Marine Biological Laboratory
LITT, MORTIMER, Instructor in Bacteriology, Harvard Medical School
LOWENHAUPT, BENJAMIN, Research Associate, Rockefeller Institute for Medical Research
LUBIN, MARTIN, Assistant Professor of Pharmacology, Harvard Medical School
McELROY, W. D., Chairman, Department of Biology, Johns Hopkins University
MARSLAND, DOUGLAS, Professor of Biology, Washington Square College, New York University
MATEYKO, G. M., Assistant Professor of Biology, Washington Square College, New York University
MERRIAM, ROBERT W., Associate in Zoology, University of Pennsylvania
METZ, CHARLES B., Professor, Florida State University
METZ, CHARLES W., Research Professor, University of Pennsylvania

Middlebrook, Robert, Associate Researcher, Institute for Muscle Research, Marine Biological Laboratory
Monroy, Alberto, Professor of Comparative Anatomy, University of Palermo
Moore, John W., Associate Chief, Laboratory of Biophysics, National Institutes of Health
Mueller, Helmut, Associate Researcher, Institute for Muscle Research, Marine Biological Laboratory
Mullins, L. J., Associate Professor of Biophysics, Purdue University
Nace, Paul Foley, Associate Professor of Zoology, McMaster University
Nelson, Leonard, Assistant Professor of Anatomy, University of Chicago
Nickerson, Norton H., Instructor in Botany, Cornell University
Norris, William Elmore, Jr., Professor of Biology, Southwest Texas State Teachers College
Odum, Howard T., Director, Institute of Marine Science, The University of Texas
Osterhout, W. J. V., Member *Emeritus,* Rockefeller Institute for Medical Research
Padawer, Jacques, Assistant Professor of Biochemistry, Albert Einstein College of Medicine
Parker, Johnson, Assistant Professor in Plant Physiology, Yale School of Forestry
Parpart, Arthur K., Professor and Chairman of Biology, Princeton University
Paterson, Mabel C., Assistant Professor of Zoology, Vassar College
Person, Philip, Chief, Special Dental Research Program, Veterans Administration Hospital
Potter, David D., Johns Hopkins University School of Medicine
Preston, James B., Assistant Professor of Physiology, State University of New York at Syracuse
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Read, Clark P., Associate Professor of Parasitology, Johns Hopkins University
Rebhun, Lionel I., Assistant Professor of Anatomy, University of Illinois
Rieser, Peter, Research Associate, University of Pennsylvania
Rockstein, Morris, Associate Professor of Physiology, New York University-Bellevue Medical Center
Rose, S. Meryl, Professor of Zoology, University of Illinois
Rosenberg, Evelyn K., Associate Professor, New York University-Bellevue Medical Center
Rosenthal, Theodore B., Assistant Professor of Anatomy, University of Pittsburgh
Roslansky, John D., Research Associate, Princeton University
Rugh, Roberts, Associate Professor of Radiology, Columbia University
Ryther, J. H., Marine Biologist, Woods Hole Oceanographic Institution
Sakai, Toshio, Research Fellow, Rockefeller Institute for Medical Research
Sanborn, Richard C., Assistant Professor of Zoology, Purdue University
Sandeen, Muriel I., Assistant Professor of Zoology, Duke University
Saunders, John W., Jr., Professor of Zoology, Marquette University
Schechter, Victor, Associate Professor of Biology, City College of New York
Schneiderman, Howard A., Associate Professor of Zoology, Cornell University
Schneller, Sister Mary Beatrice, Professor of Biology, St. Joseph College for Women
Schuh, Rev. Joseph E., Chairman, Biology Department, Saint Peter's College
Scott, Allan, Chairman, Department of Biology, Colby College
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College
Scott, George T., Professor of Zoology, Oberlin College
Segal, John R., Graduate Student, Massachusetts Institute of Technology
Senft, Alfred W., Woods Hole, Massachusetts
Shaw, Evelyn, Research Associate, American Museum of Natural History
Slifer, Eleanor H., Professor of Zoology, State University of Iowa
Smith, Paul Ferris, Electronics Engineer, Rockefeller Institute for Medical Research
Speidel, Carl C., Professor of Anatomy, University of Virginia
Spiegel, Melvin, Assistant Professor of Biology, Colby College
Spratt, Nelson T., Professor of Zoology, University of Minnesota
Spyropoulos, Constantine S., Neurophysiologist, National Institutes of Health
Starr, Richard C., Associate Professor of Botany, Indiana University
Steinbach, H. Burr, Chairman, Department of Zoology, University of Chicago
Steinhardt, Jacinto, Director, Operations Evaluation Group, Massachusetts Institute of Technology
Stephens, Grover C., Assistant Professor of Zoology, University of Minnesota

STOKEY, ALMA G., Professor *Emeritus* Mount Holyoke College
STONE, WILLIAM, Director, Ophthalmic Plastics Laboratory, Massachusetts Eye and Ear Infirmary
STROHMAN, RICHARD C., Assistant Professor of Zoology, University of California
STUNKARD, HORACE W., Research Scientist, U. S. Fish and Wildlife Service
STURTEVANT, A. H., Thomas Hunt Morgan Professor of Genetics, California Institute of Technology
SUDAK, FREDERICK N., Instructor, Albert Einstein College of Medicine
SUSSMAN, MAURICE, Associate Professor, Biology Department, Northwestern University
SZENT-GYÖRGYI, Albert, Chief Investigator, Institute for Muscle Research, Marine Biological Laboratory
SZENT-GYÖRGYI, Andrew G., Investigator, Institute for Muscle Research, Marine Biological Laboratory
SZENT-GYÖRGYI, Eva, Associate Researcher, Institute for Muscle Research, Marine Biological Laboratory
TALEPOROS, PLATO, Postdoctoral Research Fellow, University of California
TASAKI, ICHIJI, Chief, Special Senses Section, Laboratory of Neurophysiology, National Institutes of Health
TAYLOR, ROBERT E., Physiologist, National Institutes of Health
TAYLOR, WILLIAM RANDOLPH, Professor of Botany, University of Michigan
TOBIAS, JULIAN M., Professor of Physiology, University of Chicago
TODD, ROBERT E., Professor of Zoology, Colgate University
TRAUTWEIN, WOLFGANG, Associate Professor of Physiology, Johns Hopkins Hospital
TRENDELENBURG, ULLRICH, Associate in Pharmacology, Harvard Medical School
TRINKAUS, J. P., Associate Professor of Zoology, Yale University
TROLL, WALTER, Assistant Professor of Zoology, New York University-Bellevue Medical Center
TWEEDELL, KENYON S., Assistant Professor of Zoology, University of Maine
TYLER, ALBERT, Professor of Embryology, California Institute of Technology
DEVILLAFRANCA, GEORGE W., Assistant Professor of Zoology, Smith College
VINCENT, WALTER S., Assistant Professor of Anatomy, State University of New York, Upstate Medical Center, at Syracuse
WAGNER, CAPTAIN HENRY G., Head, Physiology Division, Naval Medical Research Institute
WAINIO, WALTER W., Associate Professor of Biochemistry, Rutgers University
WEBB, H. MARGUERITE, Research Associate, Northwestern University
WHITING, P. W., Professor of Zoology *Emeritus,* University of Pennsylvania
WICHTERMAN, RALPH, Professor of Biology, Temple University
WILBER, CHARLES G., Chief, Comparative Physiology Branch, Army Chemical Center
WILLEY, C. H., Professor and Chairman of Biology, New York University, University College
WILSON, WALTER L., Assistant Professor of Physiology and Biophysics, College of Medicine, University of Vermont
WITTENBERG, JONATHAN B., Assistant Professor of Physiology and Biochemistry, Albert Einstein College of Medicine
WRIGHT, PAUL A., Associate Professor of Zoology, University of Michigan
WURZEL, MENACHEM, Research Worker, College of Physicians & Surgeons, Columbia University
ZWEIFACH, BENJAMIN W., Associate Professor of Pathology, New York University—Bellevue Medical Center
ZWILLING, EDGAR, Associate Professor of Genetics, University of Connecticut

LALOR FELLOWS, 1958

AFZELIUS, B., Johns Hopkins University
BETTELHEIM, F., Adelphi College
CZERLINSKI, G., Max-Planck Inst. Physikal Chemie
ECCLES, ROSAMOND, Australian National University
HARDING, CLIFFORD V., University of Pennsylvania
KLUSS, BYRON C., Albion College
LASH, JAMES, University of Pennsylvania
LUBIN, MARTIN, Harvard Medical School
NELSON, LEONARD, University of Chicago
WITTENBERG, JONATHAN B., Albert Einstein College of Medicine

Lillie Fellow, 1958

Monroy, Alberto, University of Palermo, Italy

Grass Fellows, 1958

Bennett, Michael V. L., Columbia University
Reuben, John, University of Florida
Rickles, William H., Jr., Baylor University, College of Medicine

American Philosophical Fellows

Choi, Ki-Chul, Seoul National University, Korea
Grodzinski, F., University of Krakow, Poland
Monroy, Alberto, University of Palermo, Italy

Beginning Investigators, 1958

Ashton, Francis T., University of Pennsylvania
Braverman, Maxwell H., University of Illinois
Brett, William J., Indiana State Teachers College
Cagle, Julien, Princeton University
Dubnau, David A., Columbia University
Elliott, Paul R., University of Michigan
Feldherr, Carl, University of Pennsylvania
Fielden, Ann, University of Illinois
Frankel, Joseph, Yale University
Friz, Carl T., University of Minnesota
Fujimori, Eiji, University of Tokyo
Griffin, Joe L., Princeton University
Hathaway, Ralph R., Florida State University
Jackson, James A., Western Reserve University
Johnson, Sister Maria Benigna, Saint Joseph College
Kahlbrock, Margit M., Columbia University
Kerenyi, Thomas, Cornell Medical College
Krassner, Stuart, Johns Hopkins University
Lambert, Loretta, Harvard University
McCluskey, Robert T., New York University College of Medicine
Maturana, Humberto R., Harvard University
Moore, Richard Davis, Purdue University
Nagler, Arnold L., New York University-Bellevue Medical Center
Ralph, Charles L., U. S. Dept. of Agriculture
Rhodes, William C., Johns Hopkins University
Rickles, William H., Jr., Baylor University College of Medicine
Ross, Samuel M., State University of New York at Brooklyn
Rothman, Alvin H., Johns Hopkins School of Hygiene and Public Health
Shirodkar, Manohar V., Johns Hopkins School of Hygiene and Public Health
Sjodin, Raymond A., Purdue University
Steinberger, William W., University of Michigan
Strehler, B. L., National Institutes of Health
Swett, John Emery, University of California
Villegas, Raimundo, Harvard Medical School
Wallace, Robin A., Columbia University
Werman, Robert, Columbia University, College of Physicians and Surgeons
Werntz, Henry O., Harvard University
Wylie, Richard M., Harvard University
Young, Robert R., Harvard Medical School

Research Assistants, 1958

Adler, Howard, Albert Einstein Medical School
Adler, Julius, Washington University School of Medicine
Allen, Archie, University of North Carolina
Alsup, Peggy Ann, University of Pennsylvania
Arnold, Elizabeth, Indiana University
Auclair, Walter, New York University
Barnwell, Franklin, Northwestern University
Bedford, Bonnie V., Vassar College
Bensam, Bertrand, State University of New York Medical College at Syracuse
Birky, Carl William, Jr., Indiana University
Bischoff, Harry W., Texas Lutheran College
Borgese, Thomas A., Rutgers University
Bosler, Robert B., Johns Hopkins University School of Medicine
Briskow, Cornelia, Barnard College
Bunim, Lesley S., Barnard College
Camougis, George, University of California
Cantor, Marvin H., Massachusetts Institute of Technology
Caranasos, George J., St. Peter's College
Chesebrough, Carolyn, Mount Holyoke College
Clark, Eloise E., University of California
Clark, Lenora M., Lilly Research Laboratories
Clark, Lynne G., Queens College
Close, Russell I., University of Illinois
Cohen, Janice, New York University, College of Medicine
Cohen, Stafford I., Boston University Medical School
Cole, Ellen L., University of Pennsylvania
Conway, Dorothy M., Rockefeller Institute for Medical Research
Coutinho, Elsimar M., Faculdade de Medicina Universidade de Bahia, Brazil
Davis, Ann, Columbia University
Deason, Temd R., University of Texas
Dingle, Al D., McMaster University
Doolittle, Russell F., Harvard University
Ein, Daniel, New York University-Bellevue Medical Center
Erdman, Howard E., North Carolina State College
Esper, Hildegard, Reed College
Fine, Albert S., Veterans Administration Hospital
Frank, Colin H., Belmont High School
Friedman, Leonard, Rutgers University
Fulton, Chandler M., Rockefeller Institute
Gebhart, John H., National Institutes of Health
Gifford, Cameron E., Harvard University
Gould, Edwin, Harvard University
Green, Jonathan, University of Minnesota
Greenberg, Alan, Albert Einstein College of Medicine
Greenlees, Janet, Rutgers University
Gross, Marcie, Yale University
Grupp, Erica, Columbia University
Guttman, Burton S., University of Minnesota
Hamburger, Carola L., St. Louis
Hicks, Mary, Rockefeller Institute
Holtzman, Eric, Columbia University
Humphreys, Tom D., University of Chicago
Jones, Raymond, England
Kagey, Karen, New York University College of Medicine
Kanungo, Madhu S., University of Illinois
Kent, Joan L., Columbia University

deLannurien, Anne, Smith College
Laurie, John S., Tulane University
Lehv, Jane W., Vassar College
Lorenzo, Michael A., St. Louis University
Love, David S., University of Chicago
Lowe, Mildred E., Tulane University
Lowe, Ruth N., New York University-Bellevue Medical Center
McCann, Frances V., Columbia University
McCann, Marjorie, Louisiana State University
McLaughlin, Jane, Institute for Muscle Research, Marine Biological Laboratory
MacMullen, Joyce A., Cornell University
Malkoff, Donald B., University of Pittsburgh School of Medicine
Margolis, Phyllis, Barnard College
Mingioli, Elizabeth S., Harvard Medical School
Morgan, Miriam, Smith College
Mosher, Carter G., Boston University School of Medicine
Moule, Margaret, McMaster University
Murrell, Leonard R., McMaster University
Nass, Sylvan, New York University
Noel, Elisabeth S., Seton Hill College
Oberpriller, John, University of Illinois
Otero, Luis R., University of Puerto Rico
Paliwal, Ripusudan L., University of Oklahoma
Palmieri, Amelia, Cornell University Medical College
Pearse, John S., University of Chicago
Philpott, Delbert, Institute for Muscle Research, Marine Biological Laboratory
Plumb, Mary E., North Carolina State College
Robertson, Lola, Fish and Wildlife Service
Rodenberg, Jeannette M., New York University-Bellevue Medical Center
Rosenblum, William, New York University-Bellevue Medical Center
Rosenbluth, Raja, Columbia University
Rosillo, Ludwig, St. Peter's College
Rubinoff, Ira, American Museum of Natural History
Salach, James I., University of Chicago
Saturen, Janice R., Syracuse Medical School
Schinski, Robert A., University of Minnesota
Schuel, Herbert, University of Pennsylvania
Shepard, David, University of Chicago
Sherman, Irwin W., Northwestern University
Siger, Alvin, Johns Hopkins University
Simmons, John E., Johns Hopkins University
Spyrides, George J., Johns Hopkins University
Staub, Herbert W., Rutgers University
Sundararaj, B. I., Tulane University
Swope, Julia C., Massachusetts General Hospital
Szent-Györgyi, Martha, Institute for Muscle Research, Marine Biological Laboratory
Tsuk, Marianne, Smith College
Wahl, Rosemarie, University of Chicago
Walters, C. Patricia, Lilly Research Laboratories
Warwick, Anne C., Johns Hopkins School of Hygiene
Whitcomb, Ernest R., National Institutes of Health
Wilber, John F., Harvard Medical School
Wilbois, Annette, Indiana University
Wong, Edward T., University of Minnesota
Wood, Robert W., Sloan-Kettering Institute
Woods, B. Louise, Lilly Research Laboratories
Wyttenbach, Charles R., Carnegie Institution of Washington
Yip, Cecil, McMaster University

Library Readers, 1958

Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Batham, Elizabeth J., Lecturer, University of Otago, New Zealand
Baylor, Martha B., Investigator, Marine Biological Laboratory
Beidler, Lloyd M., Professor of Physiology, Florida State University
Bodansky, Oscar, Professor of Biochemistry, Sloan-Kettering Institute
Bridgman, Anna Josephine, Professor of Biology, Agnes Scott College
Broberg, Patricia L., Postdoctoral Fellow, Brandeis University
Browne, L. Barton, Johns Hopkins University
Butler, Elmer G., Professor of Zoology, Princeton University
Chanutin, Alfred, Professor of Biochemistry, University of Virginia
Clark, Eliot R., Professor *Emeritus* of Anatomy, University of Pennsylvania
Claudatus, John C., Head, Department of Biochemistry, The Cancer Institute at Miami
Cohen, Seymour S., Professor of Biochemistry, University of Pennsylvania
DuBois, Arthur, Associate Professor of Physiology, University of Pennsylvania
Eisen, Herman N., Professor of Dermatology, Washington University
Feenberg, Eugene, Professor of Physics, Washington University
Fries, Erik F. B., Associate Professor, City College of New York
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Gaffron, Hans, Professor of Biochemistry, University of Chicago
Ginsberg, Harold S., Associate Professor of Preventive Medicine, Western Reserve University
Gudernatsch, Frederick, Director, Cornell University Medical College
Hackett, David P., Associate Professor of Biology, University of Buffalo
Himmelfarb, Sylvia, Instructor in Physiology, University of Maryland Medical School
Hoberman, H. D., Professor of Biochemistry, Albert Einstein College of Medicine
Horsfall, Frank L., Vice-President and Physician-in-Chief, Rockefeller Institute
Jacobs, M. H., Professor of General Physiology *Emeritus,* University of Pennsylvania
Jones, Sarah R., Instructor in Zoology, Connecticut College
Kaan, Helen W., Indexer, National Academy of Sciences
Kabat, Elvin A., Professor of Microbiology, Columbia University
Karush, Fred, Professor of Immunochemistry, University of Pennsylvania School of Medicine
Keosian, John, Professor of Biology, Rutgers University
Kindred, James E., Professor of Anatomy, University of Virginia
Klein, Morton, Professor of Microbiology, Temple University School of Medicine
Lazzarini, Abel A., Associate Professor of Research Surgery, New York University
Lionetti, Fabian J., Associate Professor of Biochemistry, Boston University School of Medicine
Lochhead, John H., Professor of Zoology, University of Vermont
Lowenstein, Otto, Research Associate in Ophthalmology, Columbia University
McDonald, Sister Elizabeth Seton, Chairman, Department of Biology, College of Mt. St. Joseph
Marfey, S. Peter, Research Associate, Princeton University
Marshak, Alfred, Marine Biological Laboratory
Mavor, James, Professor *Emeritus,* Union College
Moul, Edwin T., Associate Professor of Botany, Rutgers University
Overton, Jane H., Assistant Professor of Natural Sciences, University of Chicago
Price, Winston H., Associate Professor of Epidemiology and Biochemistry, Johns Hopkins University, School of Hygiene and Public Health
Pullman, Bernard, Professor of Theoretical Chemistry, University of Paris
Roth, Jay S., Associate Professor of Biochemistry, Hahnemann Medical College
Sonnenblick, B. P., Professor of Biology, Rutgers University
Sulkin, S. Edward, Professor and Chairman, University of Texas Southwestern Medical School
Swanson, Carl P., Gill Professor in Biology, Johns Hopkins University
Trurnit, Hans J., Principal Scientist, Research Institute for Advanced Studies
Uziel, Mayo, Instructor in Biochemistry, Tufts University Medical School
Wheeler, George Edward, Instructor in Biology, Brooklyn College

Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zinn, Donald J., Associate Professor of Zoology, University of Rhode Island

Students, 1958

BOTANY

Biebel, Paul J., Indiana University
Cumming, Kenneth B., Harvard University Graduate School
Dawson, William A., Harvard University
Farquharson, Lois I., Franklin College
Fletcher, Joyce V., Cornell University
Garnett, Ellen M., Indiana University
Gibbs, Sarah P., Woods Hole
Goldstein, Melvin E., Indiana University
Goodwin, Mary Linder, Radcliffe College
Grillo, Ramon S., Fordham University
Hancock, Kenneth F., University of Alabama
Hoffman, Larry Ronald, Iowa State College
Kaushik, Nilima, Vassar College
Middleton, Katherine, Vassar College
Moran, Marius R., Fordham University
Morrill, Joy F., University of Alabama
Mumford, F. Joyce, Smith College
Ropes, Marian C., Radcliffe College

EMBRYOLOGY

Blanchard, Ann M., State University of Iowa
Boass, Agna, Radcliffe College
Cahn, Robert D., Rockefeller Institute
Corlette, Sally L., University of Pennsylvania, Institute for Cancer Research
Crowell, Jane, Radcliffe College
DiBerardino, Marie A., University of Pennsylvania, Institute for Cancer Research
Finch, Cynthia L., Oberlin College
Hunter, Roy, Jr., Brown University
Kaighn, Morris E., Massachusetts Institute of Technology
Mindich, Leonard E., Rockefeller Institute
Roberts, B. DeWayne, Roswell Park Memorial Institute
Roth, Willard D., Harvard Medical School
Sergent, Dorothy J., Mount Holyoke College
Siegel, Paula H., University of Rochester
Sonneborn, David R., Rockefeller Institute
Thompson-Upham, A. E., Amherst College
Troffkin, Walter H., Brooklyn College
Tumasonis, Rev. Casimir, Fordham University
Vanable, Joseph W., Brown University
Vining, George Joseph, Yale University
Weiss, Leon P., Harvard Medical School
Weston, Charles R., Princeton University

PHYSIOLOGY

Ascheim, Emil, New York University
Axelrod, David, Harvard Medical School
Beaulnes, Aurele, University of Montreal

Cardell, Robert R., Jr., University of Virginia
Cegler, Anneliese M., Marquette University
Curtis, Brian A., University of Rochester
Czerlinski, Georg H., Max Planck Inst. physikal Chemie
Demovsky, Ronald A., University of Illinois, College of Medicine
Elliott, Paul R., University of Michigan
Faust, Robert G., Princeton University
Ferrans, Victor J., Tulane Medical School
Franzen, James S., University of Illinois
Goldberg, Edward, Johns Hopkins University
Goyer, Robert A., St. Louis University
Griffin, Joe Lee, Princeton University
Haselkorn, Robert, Harvard University
Hoebel, Bart, Rockefeller Institute
Hutton, Kenneth, C., San Jose State College
Krause, Robert L., Haverford College
Marks, William B., Massachusetts Institute of Technology
Novick, Richard P., New York University
Roller, Ann, California Institute of Technology
Rosenkranz, Herbert, Sloan-Kettering Institute
Rueckert, Roland R., McArdle Memorial Institute, University of Wisconsin
Trygstad, Carl W., University of Florida
Weisberg, Robert A., Harvard College
Wylie, Richard M., Harvard University

INVERTEBRATE ZOOLOGY

Ashman, Robert F., Wabash College
Barry, Cornelius, University of Maryland
Bay, Ernest C., Cornell University
Birky, Carl William, Jr., Indiana University
Blank, Fenja, City College of New York
Broseghini, Albert L., Iowa State College
Cardell, Robert R., Jr., University of Virginia
Clark, James M., Franklin and Marshall College
Cooper, Edwin L., Atlanta University
Davis, Robert P., Cornell University
Dawson, Richard G., Shawnee-Mission High School
Drainville, Father Gérard, Université de Montréal
Dunagan, Tommy T., Purdue University
Eldridge, Peter J., University of Massachusetts
Fernow, Leonard R., Cornell University
Figge, Rosalie A., Oberlin College
Fiske, Timothy, University of Minnesota
Forrest, Helen F., Rutgers University
Fox, Sister M. Alice Marie, Saint Louis University
Gold, Kenneth, New York University
Gould, Edwin, Tulane University
Grant, David C., College of Wooster
Gussin, Arnold Ely, Tulane University
Guttman, Burton S., University of Minnesota
Hall, Donald J., University of Michigan
Harrer, Lora, Marquette University
Hennen, Sally H., Indiana University
Hilty, Carol R., Oberlin College
Kelso, Judith I., Brown University

Kroeckel, Rev. Clarence J., St. Joseph's College
Leary, Donald E., Notre Dame
Leet, Rosemary, Chatham College
Licht, Paul, Washington University
Lindsay, David T., Johns Hopkins University
Luning, Anne, Vassar College
Macior, Fr. Lazarus, University of Wisconsin
MacMullen, Joyce, Cornell University
McCreash, Arthur H., Temple University
McRitchie, Robert G., Vanderbilt University
Paine, Robert T., III, University of Michigan
Peel, Rosemary E., Drew University
Pernaa, Judith E., Cornell University
Robinson, Martha A., Oberlin College
Roth, Thomas F., Harvard University
Sanchez, Patricio, Rockefeller Foundation
Savage, Alice M., Brown University
Schmidt, Rev. Matthias, St. Benedict's College
Smith, Willie R., Fordham University
Snow, Isabel W., Hunter College
Stilwell, Shirley E., Wheaton College
Tunnock, Sheila M., Colby College
Vaharu, Tiiu, Syracuse University
Weston, James A., Yale University
Wetzel, Bruce K., Harvard University
Williams, Deborah C., Tufts University

ECOLOGY

Beyers, Robert J., University of Texas
Choi, Ki-Chul, Seoul National University
Cumming, Kenneth B., Harvard University
Fonda, Shirley L., Oberlin College
Goudsmit, Esther M., University of Michigan
Hochman, Robert A., Lafayette College
Moll, Carolyn J., Mount Holyoke College
Mueller, Wayne Paul, Indiana University
Patchen, Joan D., Drew University
Rosen, Donn Eric, American Museum of National History
Scully, Margaret A., Framingham State Teachers College
Sewall, Jane, Goucher College
Simcox, Richard F., Los Angeles State College
Stagner, Marilyn L., Duke University
Williams, Richard B., Harvard University
Wilson, Ronald F., Dartmouth College

3. Fellowships and Scholarships, 1958

Lucretia Crocker Scholorship:
Larry R. Hoffman, Botany Course
Calkins Scholarship:
Donald J. Hall, Invertebrate Zoology Course
Paula Siegel, Embryology Course
Walter Troffkin, Embryology Course
Bio Club Scholarship:
Fenja Blank, Invertebrate Zoology

4. TABULAR VIEW OF ATTENDANCE, 1954–58

	1954	*1955*	*1956*	*1957*	*1958*
INVESTIGATORS—TOTAL	298	250	304	326	410
Independent	180	162	184	186	203
Under Instruction	20	9	20	23	39
Library Readers	52	54	50	42	54
Research Assistants	46	25	50	75	114
STUDENTS—TOTAL	134	148	140	139	138
Invertebrate Zoology	56	56	55	55	55
Embryology	29	30	28	27	22
Physiology	28	30	30	30	27
Botany	12	19	18	18	18
Ecology	9	13	9	9	16
TOTAL ATTENDANCE	432	398	444	465	548
Less persons represented as both investigators and students	5		2	3	5
	427	398	442	462	543
INSTITUTIONS REPRESENTED—TOTAL	136	129	130	129	142
By Investigators	104	95	97	94	110
By Students	32	34	33	35	74
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	2	3	3	5	
By Students	1	2	1	1	2
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	11	8	9	11	20
By Students	13	6	6	5	6

5. INSTITUTIONS REPRESENTED, 1958

Adelphi College
Agnes Scott College
Agricultural Research Center
Alabama, University of
Albert Einstein Medical School
Albion College
American Heart Association
American Museum of Natural History
Amherst College
Army Chemical Center
Atlanta University
Barnard College
Baylor University
Boston University School of Medicine
Brandeis University
Brooklyn College
Brown University
Bryn Mawr College
Buffalo, University of
California Institute of Technology
California, University of
Cancer Institute of Miami
Carnegie Institute of Technology
Carnegie Institution of Washington
Chatham College
Chicago, University of
Cincinnati, University of
City College of New York
Colby College
Colgate University
Colorado, University of
Columbia University
Columbia University College of Physicians and Surgeons
Connecticut College
Connecticut, University of
Cornell University
Cornell University Medical School
Dartmouth College
Drew University
Duke University
Eli Lilly and Company
Emory University
Florida State University
Fordham University
Framingham State Teachers College
Franklin College
Franklin and Marshall College

Goucher College
Hahnemann Medical School
Harvard University
Harvard University Medical School
Haverford College
Howard Hughes Medical Institute
Hunter College
Illinois, University of
Indiana State Teachers College
Indiana University
Institute for Muscle Research
Iowa State University
Johns Hopkins University
Lafayette College
Los Angeles State College
Louisiana State University
Maine, University of
Marquette University
Maryland, University of
Mass. Eye and Ear Infirmary
Mass. General Hospital
Mass. Institute of Technology
Massachusetts, University of
Michigan, University of
Minnesota, University of
Mount Holyoke College
Mt. St. Joseph, College of
National Academy of Sciences
National Institutes of Health
Naval Medical Research Institute
New York, State University of, Medical School at Syracuse
New York, State University of, at Brooklyn
New York University, Bellevue Medical Center
New York University, School of Dentistry
New York University, Washington Square College
North Carolina State College
North Carolina, University of
Northwestern University
Notre Dame University
Oak Ridge National Laboratory
Oberlin College
Oklahoma, University of
Oregon, University of
Pennsylvania, University of
Pennsylvania Medical School, University of
Pittsburgh, University of
Polytechnic Institute of Brooklyn
Princeton University
Purdue University
Queens College
Radcliffe College
Reed College
Research Institute for Advanced Studies
Rhode Island, University of
Rochester, University of
Rockefeller Institute for Medical Research
Roswell Park Memorial Institute
Rutgers University
St. Benedict's College
St. Joseph College for Women
St. Joseph's College
St. Louis University
St. Peter's College
San Jose State College
Seton Hall College
Seton Hill College
Sloan-Kettering Institute
Smith College
Southwest Texas State Teachers College
Syracuse University
Temple University
Texas Lutheran College
Texas, University of
Texas, University of, Southwestern Medical School
Tufts University
Tufts University Medical School
Tulane University
Tulane University Medical School
Union College
U. S. Fish and Wildlife Service
U. S. Public Health Service
Vanderbilt University
Vassar College
Vermont, University of
Veterans' Administration Hospital
Virginia, University of
Washington University
Washington University Medical School
Western Reserve University
Wheaton College
Wilson College
Wisconsin, University of
Wooster, College of
Yale University
Yeshiva University

FOREIGN INSTITUTIONS REPRESENTED, 1958

Australian National University, Australia
Universidade de Bahia, Brazil
Atomic Energy Authority, British Isles
Kings College, British Isles
University of Cambridge, British Isles
University of Reading, British Isles
McMaster University, Canada
University of Montreal, Canada
University of Chile, Chile
Ecole Scientia et Faculty de Mecidna, France

Max Planck Institut für physikalische Chemie, Germany
Physiological Institute of Heidelberg, Germany
University of Tübingen, Germany
University of Hawaii
Central College, University of Mysore, India
University of Palermo, Italy
Hebrew University, Hadassah Medical School, Jordan
Kogoshima University, Japan
Tokyo Jikei-kai School of Medicine, Japan
University of Tokyo, Japan
Seoul National Institute, Korea
University of Otago, New Zealand
Fagellonian University, Poland
University of Puerto Rico, Puerto Rico
Wenner Grens Institute, Sweden
Institute de Investigaciones Medicas, Venezuela

Supporting Institutions and Agencies, 1958

Abbott Laboratories
American Cancer Society
American Philosophical Society
Associates of the Marine Biological Laboratory
Atomic Energy Commission
Ciba Pharmaceutical Products, Inc.
Josephine B. Crane Foundation
Carter Products Inc.
The Grass Foundation
Hoffman-LaRoche, Inc.
The Lalor Foundation
Eli Lilly and Company
Merck and Company, Inc.
National Institutes of Health
National Science Foundation
Office of Naval Research
The Pfizer Foundation, Inc.
The Rockefeller Foundation
Schering Corporation
Smith, Kline and French Foundation
Wyeth Laboratories
The Upjohn Company

6. Friday Evening Lectures, 1958

July 4	Albert Szent-Györgyi	"Muscle and energetics"
July 11	Colin S. Pittendrigh	"A coupled oscillator scheme for the daily rhythms of organisms"
July 18	Bernard D. Davis	"Bacterial mutants and the study of cell physiology"
July 25	Warren O. Nelson	"The physiological control of fertility"
August 1	Rollin D. Hotchkiss	"The dissemination of genetic substance"
August 8	Stephen W. Kuffler	"A single nerve cell looks at neurophysiology"
August 15	Don W. Fawcett	"The submicroscopic structure and functional behavior of the membranous components of the cytoplasm"
August 22	Bernard Pullman	"Electronic structure and activity in cancer chemotherapy of purine antimetabolites"

7. Tuesday Evening Seminars, 1958

July 1	Edgar Zwilling	"Reconstitution from one germ layer in Cordylophora (hydroid)"

MAXWELL BRAVERMAN	"Neural and mesodermal hierarchies in chick development"
S. MERYL ROSE	"Mutual growth inhibition in frog tadpoles"
July 8	
FREDERICK A. BETTELHEIM	"The nature of chromatographic amylose and amylopectin fractions"
PAUL S. GALTSOFF	"Coordination of ciliary motion and muscular activity in *Ostrea virginica*"
BENJAMIN LOWENHAUPT	"The carrier for calcium transport in aquatic leaves"
July 15	
P. W. WHITING	"Factors and genes in Mormoniella"
SEARS CROWELL	"Tail regeneration in experimentally shortened and lengthened earthworms"
C. C. SPEIDEL	"Motion pictures showing some changes in cells induced by x-ray treatments of tadpoles and tetrahymenae"
July 22	
JAMES LASH	"The uptake of radiosulphur during the induction of cartilage"
MENACHEM WURZEL	"Mode of action of choline esters, substrate specificity of their 'receptor protein' "
IRVIN ISENBERG	"Free radical formation in riboflavin complexes"
July 29	
A. J. BERNATOWICZ	"Ecological isolation of alternate generations of plants"
DEMOREST DAVENPORT	"A technique of investigating the effect of host-factor on the behavior of polychaete and crustacean commensals"
EVELYN SHAW	"The development of schooling behavior in the silverside fish, *Menidia menidia*"
CHARLES JENNER	"Schooling behavior in the marine snail, *Nassarius obsoletus*" (with colored movie)
August 5	
ARTHUR L. COLWIN and LAURA H. COLWIN	"Effects of sperm extract and other agents on the egg membranes, in relation to sperm entry in Hydroides"
CHARLES B. METZ	"Fertilization and agglutination inhibitors from Arbacia"
LIONEL I. REBHUN	"Behavior of metachromatic granules during cleavage in Spisula"
August 12	
G. W. DE VILLAFRANCA, T. S. SCHEINBLUM and D. E. PHILPOTT	"The a-band of muscle from *Limulus polyphemus*"
D. W. BISHOP	"Sperm cell models and the question of ATP-induced rhythmic motility"
L. NELSON	"ATP—An energy source for sperm motility"

R. D. Allen	"Polarized optical studies on Ameba"
F. Child	"Isolation and analysis of cilia"
August 19	
L. V. Heilbrunn	"A physical study of the ground substance of the Spisula egg"
Carl Feldherr	"Physical properties of lobster nerve axoplasm"
Robert W. Merriam	"Some aspects of the nuclear membrane in developing sand dollar eggs"

8. Members of the Corporation, 1958

1. Life Members

Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania
Carver, Dr. Gail L., Mercer University, Macon, Georgia
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri
Crane, Mrs. W. Murray, Woods Hole, Massachusetts
Dederer, Dr. Pauline H., Connecticut College, New London, Connecticut
Goldfarb, Dr. A. J., College of the City of New York, New York City, New York
Knowlton, Dr. F. P., 1356 Westmoreland Avenue, Syracuse, New York
Lewis, Dr. W. H., Johns Hopkins University, Baltimore, Maryland
Lowther, Dr. Florence del., Barnard College, New York City, New York
MacNaught, Mr. Frank M., Woods Hole, Massachusetts
Macklin, Dr. Charles C., 37 Gerard Street, London, Ontario
Malone, Dr. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts
Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pennsylvania
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania
Riggs, Mr. Lawrason, 74 Trinity Place, New York 6, New York
Scott, Dr. Ernest L., Columbia University, New York City, New York
Turner, Dr. C. L., Northwestern University, Evanston, Illinois
Waite, Dr. F. G., 144 Locust Street, Dover, New Hampshire
Wallace, Dr. Louise B., 359 Lytton Avenue, Palo Alto, California
Warren, Dr. Herbert S., 610 Montgomery Avenue, Bryn Mawr, Pennsylvania
Young, Dr. B. P., Cornell University, Ithaca, New York

2. Regular Members

Abell, Dr. Richard G., 7 Cooper Road, New York City, New York
Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts
Addison, Dr. W. H. F., 286 East Sidney Avenue, Mount Vernon, New York
Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, New York
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota
Allen, Dr. M. Jean, Department of Biology, Wilson College, Chambersburg, Pennsylvania

Allen, Dr. Robert D., Department of Biology, Princeton University, Princeton, New Jersey

Alscher, Dr. Ruth, Department of Physiology, Manhattanville College, Purchase, New York

Amberson, Dr. William R., Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

Anderson, Dr. J. M., Department of Zoology, Cornell University, Ithaca, New York

Anderson, Dr. Rubert S., Medical Laboratories, Army Chemical Center, Maryland (Box 632 Edgewood, Maryland)

Anderson, Dr. T. F., c/o Dr. A. Lurff, Institut Pasteur, 28 Rue du Dr. Roux, Paris 15e, France

Armstrong, Dr. Philip B., State University of New York College of Medicine, Syracuse 10, New York

Arnold, Dr. William A., Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Atwood, Dr. Kimball C., 6029 University Avenue, Chicago 37, Illinois

Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts

Ayers, Dr. John C., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Baitsell, Dr. George A., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

Baker, Dr. H. B., Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania

Ball, Dr. Eric G., Department of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire

Bang, Dr. F. B., Department of Pathobiology, Johns Hopkins University School of Hygiene, Baltimore 5, Maryland

Bard, Dr. Philip, Johns Hopkins Medical School, Baltimore, Maryland

Barth, Dr. L. G., Department of Zoology, Columbia University, New York 27, New York

Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois

Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa

Beck, Dr. L. V., Department of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

Beers, Dr. C. D., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Behre, Dr. Elinor H., Black Mountain, North Carolina

Benesch, Dr. Reinhold, Marine Biological Laboratory, Woods Hole, Massachusetts

Benesch, Dr. Ruth, Marine Biological Laboratory, Woods Hole, Massachusetts

Bennett, Dr. Miriam F., Department of Biology, Sweet Briar College, Sweet Briar, Virginia

Berg, Dr. William E., Department of Zoology, University of California, Berkeley 4, California

Berman, Dr. Mones, Institute for Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland
Bernheimer, Dr. Alan W., New York University College of Medicine, New York 16, New York
Bernstein, Dr. Maurice, Department of Anatomy, Wayne University College of Medicine, Detroit 7, Michigan
Bertholf, Dr. Lloyd, Illinois Wesleyan University, Bloomington, Illinois
Bevelander, Dr. Gerrit, New York University School of Medicine, New York 16, New York
Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge 38, Massachusetts
Bishop, Dr. David W., Department of Embryology, Carnegie Institution of Washington, Baltimore 5, Maryland
Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore, Maryland
Bloch, Dr. Robert, 518 South 42nd Street, Apt. C 7, Philadelphia 4, Pennsylvania
Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey
Bodansky, Dr. Oscar, Department of Biochemistry, Memorial Cancer Center, 444 East 68th Street, New York 21, New York
Bodian, Dr. David, Department of Anatomy, Johns Hopkins University, 709 North Wolfe Street, Baltimore 5, Maryland
Boell, Dr. Edgar J., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut
Boettiger, Dr. Edward G., Department of Zoology, University of Connecticut, Storrs, Connecticut
Bold, Dr. Harold C., Department of Botany, University of Texas, Austin, Texas
Borei, Dr. Hans, Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania
Bowen, Dr. Vaughan T., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts
Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley 4, California
Bridgman, Dr. Anna J., Department of Biology, Agnes Scott College, Decatur, Georgia
Bronk, Dr. Detlev W., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York
Brooks, Dr. Matilda M., Department of Physiology, University of California, Berkeley 4, California
Brown, Dr. Dugald E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan
Brown, Dr. Frank A., Jr., Department of Biological Sciences, Northwestern University, Evanston, Illinois
Brownell, Dr. Katherine A., Department of Physiology, Ohio State University, Columbus, Ohio
Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda 14, Maryland
Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia

Bullock, Dr. T. H., Department of Zoology, University of California, Los Angeles 24, California

Burbanck, Dr. William D., Box 721, Woods Hole, Massachusetts

Burdick, Dr. C. Lalor, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware

Burkenroad, Dr. M. D., c/o Lab. Nal. de Pesca, Apartado 3318, Estofeta #1, Olindania, Republic of Panama

Butler, Dr. E. G., Department of Biology, P.O. Box 704, Princeton University, Princeton, New Jersey

Cameron, Dr. J. A., Baylor College of Dentistry, Dallas, Texas

Cantoni, Dr. Giulio, National Institutes of Health, Mental Health, Bethesda 14, Maryland

Carlson, Dr. Francis D., Department of Biophysics, Johns Hopkins University, Baltimore 18, Maryland

Carpenter, Dr. Russell L., Tufts University, Medford 55, Massachusetts

Carson, Miss Rachel, 11701 Berwick Road, Silver Spring, Maryland

Case, Dr. James, Department of Zoology, State University of Iowa, Iowa City, Iowa

Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York City, New York

Cattell, Mr. Ware, Cosmos Club, Washington 5, D. C.

Chaet, Dr. Alfred B., Department of Biology, American University, Washington 16, D. C.

Chambers, Dr. Edward, Department of Physiology, University of Miami Medical School, Coral Gables, Florida

Chang, Dr. Joseph J., National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda 14, Maryland

Chase, Dr. Aurin M., Department of Biology, Princeton University, Princeton, New Jersey

Cheney, Dr. Ralph H., Biology Department, Brooklyn College, Brooklyn 10, New York

Claff, Dr. C. Lloyd, 5 Van Beal Road, Randolph, Massachusetts

Clark, Dr. A. M., Department of Biology, University of Delaware, Newark, Delaware

Clark, Dr. E. R., The Wistar Institute, Woodland Avenue and 36th Street, Philadelphia 4, Pennsylvania

Clark, Dr. Leonard B., Department of Biology, Union College, Schenectady, New York

Clarke, Dr. George L., Harvard University, Biological Laboratories, Cambridge 38, Massachusetts

Cleland, Dr. Ralph E., Indiana University, Bloomington, Indiana

Clement, Dr. A. C., Department of Biology, Emory University, Atlanta, Georgia

Clowes, Dr. G. H. A., Eli Lilly and Company, Indianapolis, Indiana

Coe, Dr. W. R., 183 Third Avenue, Chula Vista, California

Cohen, Dr. Seymour S., Department of Physiological Chemistry, University of Pennsylvania, Philadelphia 4, Pennsylvania

Cole, Dr. Kenneth S., National Institutes of Health (NINDB), Bethesda 14, Maryland
Collett, Dr. Mary E., 34 Weston Road, Wellesley 81, Massachusetts
Collier, Dr. Jack R., Department of Zoology, Louisiana State University, Baton Rouge, Louisiana
Colton, Dr. H. S., Box 601, Flagstaff, Arizona
Colwin, Dr. Arthur L., Department of Biology, Queens College, Flushing, New York
Colwin, Dr. Laura H., Department of Biology, Queens College, Flushing, New York
Cooper, Dr. Kenneth W., Department of Zoology, University of Florida, Gainesville, Florida
Cooperstein, Dr. Sherwin J., Department of Anatomy, Western Reserve University Medical School, Cleveland, Ohio
Copeland, Dr. D. E., 8705 Susanna Lane, Chevy Chase 15, Maryland
Copeland, Dr. Manton, Bowdoin College, Brunswick, Maine
Cornman, Dr. Ivor, Hazleton Laboratories, Box 333, Falls Church, Virginia
Costello, Dr. Donald P., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina
Costello, Dr. Helen Miller, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina
Crane, Mr. John O., Woods Hole, Massachusetts
Crane, Dr. Robert K., Department of Biological Chemistry, Washington University Medical School, St. Louis, Missouri
Croasdale, Dr. Hannah T., Dartmouth College, Hanover, New Hampshire
Crouse, Dr. Helen V., Goucher College, Towson, Baltimore 4, Maryland
Crowell, Dr. P. S., Jr., Department of Zoology, Indiana University, Bloomington, Indiana
Csapo, Dr. Arpad I., Rockefeller Institute for Medical Research, 66th Street and York Avenue, New York 21, New York
Curtis, Dr. Maynie R., University of Miami, Box 1015, South Miami, Florida
Curtis, Dr. W. C., University of Missouri, Columbia, Missouri
Dan, Dr. Jean Clark, Misaki Biological Station, Misaki, Japan
Dan, Dr. Katsuma, Misaki Biological Station, Misaki, Japan
Danielli, Dr. James F., Department of Zoology, King's College, London, England
Davis, Dr. Bernard D., Harvard Medical School, 25 Shattuck Street, Boston 15, Massachusetts
Dawson, Dr. A. B., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts
Dawson, Dr. A. J., College of the City of New York, New York City, New York
Deane, Dr. Helen W., Albert Einstein College of Medicine, New York 61, New York
Diller, Dr. Irene C., Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania
Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania
Dixon, Dr. Frank J., Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

Dodds, Dr. G. S., West Virginia University School of Medicine, Morgantown, West Virginia
Dolley, Dr. William L., Department of Biology, Randolph-Macon College, Ashland, Virginia
Donaldson, Dr. John C., University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania
Doty, Dr. Maxwell S., Department of Biology, University of Hawaii, Honolulu, T. H.
DuBois, Dr. Eugene F., 200 East End Avenue, New York 28, New York
Duryee, Dr. William R., George Washington University School of Medicine, Department of Physiology, Washington 5, D. C.
Edds, Dr. Mac V., Jr., Department of Biology, Brown University, Providence 12, Rhode Island
Edwards, Dr. Charles, University of Utah, Salt Lake City, Utah
Eichel, Dr. Herbert J., Hahnemann Medical College, Philadelphia, Pennsylvania
Elliot, Dr. Alfred M., Department of Zoology, University of Michigan, Ann Arbor, Michigan
Essner, Dr. Edward S., Department of Pathology, Albert Einstein College of Medicine, New York 61, New York
Evans, Dr. Titus C., State University of Iowa, Iowa City, Iowa
Failla, Dr. G., Columbia University, College of Physicians and Surgeons, New York 32, New York
Fauré-Fremiet, Dr. Emmanuel, Collège de France, Paris, France
Ferguson, Dr. F. P., Department of Physiology, University of Maryland Medical School, Baltimore 1, Maryland
Ferguson, Dr. James K. W., Connought Laboratories, University of Toronto, Ontario, Canada
Figge, Dr. F. H. J., University of Maryland Medical School, Lombard and Green Streets, Baltimore 1, Maryland
Fingerman, Dr. Milton, Department of Zoology, Newcomb College, Tulane University, New Orleans 18, Louisiana
Fischer, Dr. Ernst, Department of Physiology, Medical College of Virginia, Richmond 19, Virginia
Fisher, Dr. Jeanne M., Department of Biochemistry, University of Toronto, Toronto, Canada
Fisher, Dr. Kenneth C., Department of Biology, University of Toronto, Toronto, Canada
Forbes, Dr. Alexander, Biological Laboratories, Harvard University, Cambridge 38, Massachusetts
Fraenkel, Dr. Gottfried S., Department of Entomology, University of Illinois, Urbana, Illinois
Freygang, Dr. Walter H., Jr., P. O. Box 11, Poolesville, Maryland
Fries, Dr. Erik F. B., Box 605, Woods Hole, Massachusetts
Frisch, Dr. John A., Canisius College, Buffalo, New York
Furth, Dr. Jacob, 18 Springdale Road, Wellesley Farms, Massachusetts
Fye, Dr. Paul M., Director, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Gabriel, Dr. Mordecai, Department of Biology, Brooklyn College, Brooklyn 10, New York

Gaffron, Dr. Hans, Research Institutes, University of Chicago, 5650 Ellis Avenue, Chicago 37, Illinois

Gall, Dr. Joseph G., Department of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Galtsoff, Dr. Paul S., Woods Hole, Massachusetts

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Brown, Mrs. Thornton
Burdick, Dr. C. Lalor
Burlingame, Mrs. F. A.
Cahoon, Mrs. Samuel, Sr.
Calkins, Mrs. Gary N.
Calkins, Mrs. G. Nathan, Jr.
Calkins, Mr. and Mrs. Samuel W.
Carlton, Mr. and Mrs. Winslow
Claff, Dr. and Mrs. C. Lloyd
Clark, Dr. and Mrs. Alfred Hull
Clark, Mrs. Leroy
Clark, Mr. and Mrs. W. Van Alan
Clowes, Mr. Allen W.
Clowes, Mrs. G. H. A.
Clowes, Dr. and Mrs. G. H. A., Jr.
Colton, Mr. and Mrs. H. Seymour
Crane, Mr. and Mrs. Bruce
Crane, Mr. John
Crane, Miss Louise
Crane, Mrs. Murray
Crane, Mr. Stephen
Crane, Mrs. W. Carey
Cowdry, Dr. and Mrs. E. V.
Crossley, Mr. and Mrs. Archibald M.
Crowell, Mr. and Mrs. Prince S.
Curtis, Dr. and Mrs. W. D.
Daniels, Mr. and Mrs. F. Harold
Day, Mr. and Mrs. Pomeroy
Draper, Mrs. Mary C.
Dreyer, Mr. and Mrs. Frank A.
Elsmith, Mrs. Dorothy
Enders, Mr. and Mrs. Frederick
Ewing, Mr. and Mrs. Frederic
Fay, Mr. and Mrs. Henry H.
Fisher, Mr. and Mrs. B. C.
Francis, Mrs. Lewis H., Jr.
Frost, Mrs. Frank J.
Galtsoff, Mrs. Paul S.
Gifford, Mr. and Mrs. John A.
Gilchrist, Mr. and Mrs. John M.
Gildea, Dr. and Mrs. E. F.
Green, Miss Gladys M.
Haig, Mrs. R. H.
Hamlen, Mr. and Mrs. J. Monroe
Harrell, Mr. and Mrs. Joel E.
Harrington, Mr. and Mrs. Robert
Herrington, Mrs. A. W. S.
Hervey, Dr. and Mrs. John P.
Hirschfeld, Mrs. Nathan B.
Houston, Mr. and Mrs. Howard
Jewett, Mrs. G. F.
Keith, Mr. and Mrs. Harold C.
King, Mr. and Mrs. Franklin
Koller, Mr. and Mrs. Lewis
Lemann, Mrs. Benjamin
Lobb, Mrs. John
Loeb, Dr. and Mrs. Robert F.
McCusker, Mr. and Mrs. Paul J.
McKelvy, Mr. John E.
Marsland, Mrs. Douglas A.
Marvin, Mrs. Walter T.
Mast, Mrs. S. O.
Meigs, Dr. and Mrs. J. Wister
Mitchell, Mrs. James McC.
Mixter, Mrs. W. Jason
Mosser, Mrs. Benjamin D.
Motley, Mrs. Thomas
Newton, Miss Helen
Nichols, Mrs. George
Nicholson, Rev. Robert W.
Nims, Mrs. E. D.
Norman Fund, Inc., Aaron E.
Packard, Dr. and Mrs. Charles
Park, Mr. and Mrs. M. S.
Pennington, Miss Anne H.
Redfield, Dr. and Mrs. Alfred C.
Reznikoff, Dr. and Mrs. Paul
Riggs, Mr. and Mrs. Lawrason
Rivinus, Mrs. F. M., Jr.
Root, Mrs. Walter S.
Rozendaal, Dr. H. M.
Rudd, Mr. and Mrs. H. W. Dwight
Sands, Miss Adelaide G.

Saunders, Mr. and Mrs. Lawrence
Shiverick, Mrs. Arthur
Sinclair, Mr. and Mrs. W. Richardson
Speidel, Dr. and Mrs. Carl
Stockard, Mrs. Charles R.
Stone, Mr. and Mrs. Leo
Stone, Mr. and Mrs. S. M.
Straus, Dr. and Mrs. Donald B.
Swift, Mr. E. Kent
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta
Tompkins, Mr. and Mrs. B. A.
Webster, Mrs. Edwin S.
Whiteley, Miss Mabel W.
Wickersham, Mr. and Mrs. James H.
Wilhelm, Dr. and Mrs. Hilmer J.
Williston, Miss Emily
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. Wolfe

V. REPORT OF THE LIBRARIAN

In 1958, fifty-eight new journals were acquired, bringing the total number of currently received titles to 1654. Of these, there were 494 (11 new) Marine Biological Laboratory subscriptions, 621 (18 new) exchanges and 188 (9 new) gifts; 95 (5 new) were Woods Hole Oceanographic Institution subscriptions; 195 (4 new) were exchanges and 61 (11 new) were gifts.

The Laboratory purchased 145 books, received 84 complimentary copies (5 from authors and 79 from publishers) and accepted 38 miscellaneous gifts. The Institution purchased 75 titles and received 12 gifts. The total number of books accessioned totalled 354. Many books in the physical sciences were purchased, thus filling a demand that has been apparent for many years.

Through purchase, exchange and gift the Laboratory completed 8 journal sets and partially completed 32. The Institution completed 3 sets and partially completed 5. There were 3873 reprints added to the collection of which 1722 were of current issue.

At the close of the year, the Library contained 74,590 bound volumes and 209,998 reprints.

The Library sent out on inter-library loan 332 volumes and borrowed 112, a decided increase over 1957. Several copying machines were tried out, none of which met the necessary requirements. About 970 volumes and 198 pamphlets were bound.

A large reprint collection was presented by Dr. J. Percy Moore of which about 1000 papers were added to the shelves. A large percentage of the duplicate material was presented to the U. S. Fish and Wildlife Service Library and to the Library of the Narragansett Laboratory. Many zoological papers were stored for future replacement copies of articles used in the Invertebrate Course. Through Dr. Arnold Lazarow a collection was received from the University of Minnesota containing hundreds of papers published during the 19th century.

Smaller pamphlet collections were received from the Dept. of Biological Chemistry, Harvard Medical School; Dr. Benj. P. Sonnenblick, and the estate of the late Dr. Chas. R. Stockard. Mrs. A. R. Memhard presented fourteen books belonging to her late husband. Two books were received from Dr. Alfred G. Marshak; four from Dr. Henry Stommel; two from Dr. Albert Szent-Györgyi; several early Reports of the Division of Fish and Game, State of Massachusetts, from Dr. David Belding; and early numbers of the "Biological Bulletin" were returned to stock by Dr. Carl Gans and by Dr. P. W. Whiting.

The Library extends grateful acknowledgement to all of its friends who have so generously made the donations mentioned above.

During the year, some of the visiting scientists from foreign countries selected duplicate material which was shipped to the following institutions: Caribbean Marine Biological Institute, Curacäo; Faculty of Fisheries, Hokkaido University, Japan; Chulalongkorn University, Bangkok; and the New Zealand Oceanographic Institution, Wellington.

The willing assistance given by the members of the Library Committee and the Book Committee has made the year a progressive one.

Respectfully submitted,
DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE TREASURER

The market value of both the General Endowment Fund and the fund for the Library at December 31, 1958, amounted to $1,719,105 as compared with the total of $1,461,278 as of December 31, 1957. The average yield on the Securities was 3.48% of market value and 5.90% of book value. The total uninvested principal cash in the above accounts as of December 31, 1958, was $2,840. Classification of the Securities held in the Endowment Funds appears in the auditor's report.

The market value of the pooled securities as of December 31, 1958, was $297,441 with uninvested principal cash of $469; the value at December 31, 1957, being $247,629. The book value of the securities in this account was $243,958 on December 31, 1958, compared with $236,735 a year earlier. The average yield on market value was 3.64% and 4.44% of book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1958, is as follows:

Pension Funds	19.495%
General Laboratory Investment	56.540
Other:	
Bio Club Scholarship Fund	1.648
Rev. Arsenious Boyer Scholarship Fund	2.017
Gary N. Calkins Fund	1.889
Allen R. Memhard Fund	.366
F. R. Lillie Memorial Fund	6.366
Lucretia Crocker Fund	6.892
E. G. Conklin Fund	1.166
M. H. Jacobs Scholarship Fund	.831
Jewett Memorial Fund	.612
Anonymous Gift	2.178

The Jewett Memorial Fund and the Anonymous Gift Fund are included in the Pool Fund Account; however, it has not been determined how these funds are to be used.

The special Custodian account which was used to activate available funds of a temporary nature last year yielded income of $5,957, plus $1,487 interest from Savings Bank deposits of a similar nature. This income is being reserved for capital improvements.

The Securities pledged to cover the M.B.L. Club Loan matured in May, 1958. These Securities were converted into cash on deposit in a savings account with the Falmouth National Bank in the amount of $3,046. The amount of the Loan is approximately $2,000.

Donations from the M.B.L. Associates for 1958 were $3,150 as compared with $3,481 for 1957. Unrestricted gifts from foundations, societies and companies amounted to $38,579.

We are administering 21 grants for investigators in addition to those directly to the M.B.L. The amounts of the grants vary in accordance with the investigator's project of research. An amount of 15% based on the amount expended is allowed the Laboratory as overhead.

Major new construction grants awarded during 1958 are as follows:

New Laboratory Building:	
Rockefeller Institute	$ 738,500
National Institutes of Health	369,250
National Science Foundation	369,250
	$1,477,000
Devil's Lane Housing:	
National Science Foundation	$ 175,000
Grass Foundation	10,000
	$ 185,000

Lybrand, Ross Bros. & Montgomery have examined our books and submitted financial statements for examination.

Following is a statement of the auditors:

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of Marine Biological Laboratory as at December 31, 1958 and 1957, the related statements of operation expenditures and income for the years then ended, and statement of current fund for the year ended December 31, 1958. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we consider necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1958 and 1957, and the expenditures and income for the years then ended.

LYBRAND, ROSS BROS. & MONTGOMERY

Boston, Massachusetts
June 3, 1959

JAMES H. WICKERSHAM,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1958

Investments

Investments held by Trustee:	
Securities, at cost (approximate market quotation 1958—$1,719,105; 1957—$1,461,278)	$1,014,460
Cash	2,840
	1,017,300
Investments of other endowment and unrestricted funds:	
Pooled investments, at cost (approximate market quotation 1958—$297,441; 1957—$247,629)	243,958
Less temporary investment of current fund cash	5,728
	238,230
Other investments	69,756
Cash (note A)	13,401
Accounts receivable	2,435
	323,822

Plant Assets

Land, buildings, library and equipment (note B)	2,970,655
Less allowance for depreciation (note B)	1,063,577
	1,907,078
Construction in progress	156,301
Cash	9,255
Accounts receivable	11,757
U. S. Government obligations, at cost:	
$720,000 Treasury notes, 3½s, 11/15/59	722,025
	2,806,416

Current Assets

Cash	118,480
U. S. Government obligations, at cost:	
$30,000 Treasury notes, 3½s, 11/15/59	30,020
Temporary investment in pooled securities	5,728
Accounts receivable (U. S. Government, 1958—$32,808; 1957—$19,605)	52,379
Inventories of specimens and Bulletins	71,110
Prepaid insurance and other	15,383
	$4,440,638

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1958

Endowment Funds

Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,017,300
Endowment funds for awards and scholarships:	
Principal	64,415
Unexpended income	3,144
	67,559
Unrestricted funds functioning as endowment	206,378
Retirement fund	52,759
Pooled investments—accumulated gain or (loss)	(2,874)
	323,822

Plant Liability and Funds

Funds expended for plant, less retirements	3,103,854
Less allowance for depreciation charged thereto	1,063,577
	2,040,277
Unexpended plant funds	743,037
	2,783,314
Accounts payable	23,102
	2,806,416

Current Liabilities and Funds

Accounts payable	33,155
Unexpended balances of gifts for designated purposes	9,767
Advance payments on research contracts	109,792
Current fund	140,386
	$4,440,638

Notes:

A—The Laboratory has guaranteed a note of approximately $2,000 of the M.B.L. Club and has deposited as security therefor the passbook for a savings account deposit of $3,046, included in cash.

B—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Statements of Operating Expenditures and Income

Year Ended December 31, 1958

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$162,011
Instruction	39,190
Library, including book purchases	35,270
Biological Bulletin	22,050
	258,521
Direct costs on research contracts	134,566
Administration and general	61,140
Plant operation and maintenance	93,823
Dormitories and dining services	146,526
Plant additions from current funds	14,867
	709,443
Less depreciation included in plant operation and dormitories and dining services above but charged to plant funds	38,884
	670,559

Income

Direct income of departments:	
Research fees	47,269
Accessory services (including sales of biological specimens, 1958—$73,354	116,366
Instruction fees	16,865
Library fees and income	9,586
Biological Bulletin, subscriptions and sales	16,071
	206,157
Reimbursement and allowance for direct and indirect costs on research contracts	146,394
Dormitories and dining services income	108,419
	460,970
Investment income used for current expenses:	
Endowment funds	88,106
Current fund investments	7,781
Gifts used for current expenses	105,829
Sundry income	5
Total current income	662,691
Excess of income or (operating expenditures)	($ 7,868)

MARINE BIOLOGICAL LABORATORY

Statement of Current Fund

Year Ended December 31, 1958

Balance January 1, 1958	$148,254
Excess of operating expenditures over income 1958	(7,868)
Balance December 31, 1958	$140,386

MARINE BIOLOGICAL LABORATORY

Summary of Investments of Endowment Funds

December 31, 1958

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1958
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds					$ 387
Other bonds	$ 499,403	58.7	$ 479,575	33.9	15,072
	499,403	58.7	479,575	33.9	15,459
Preferred stocks	85,788	10.1	71,025	5.0	3,370
Common stocks	265,979	31.2	862,808	61.1	30,824
	851,170	100.0	1,413,408	100.0	49,653
General Educational Board endowment fund:					
U. S. Government bonds					312
Other bonds	95,004	58.2	89,525	29.3	2,963
	95,004	58.2	89,525	29.3	3,275
Preferred stocks	27,281	16.7	24,268	7.9	1,130
Common stocks	41,005	25.1	191,904	62.8	5,839
	163,290	100.0	305,697	100.0	10,244
Total securities held by Trustee	$1,014,460		$1,719,105		$59,897
Investments of other endowment and unrestricted funds:					
Pooled investments:					
Other bonds	135,666	55.6	137,816	46.3	5,201
Preferred stocks	10,531	4.3	11,350	3.8	73
Common stocks	97,761	40.1	148,275	49.9	5,547
	243,958	100.0	$ 297,441	100.0	10,821
Other investments:					
U. S. Government bonds					103
Common stocks	43,600				22,470
Real estate	26,156				167
	69,756				22,740
Total investments of other endowment and unrestricted funds	$ 313,714				$33,561
Total investment income					93,458
Custodian's fees charged thereto					(885)
Income of current funds temporarily invested in pooled securities					(220)
Investment income distributed to funds					$92,353

SOME OBSERVATIONS ON CHLAMYDOMONAS MICROHALOPHILA SP. NOV.[1]

HARRY W. BISCHOFF

Department of Biology, Texas Lutheran College, Seguin, Texas

During the summer of 1958 the writer became interested in the profuse blooms of a species of *Chlamydomonas* in some barrels of water stored at the head of the Supply Department dock, Marine Biological Laboratory, Woods Hole, Massachusetts.[2] Previous morphological and cytological work on various species of *Chlamydomonas,* especially that of Bold (1949), and Buffaloe (1958), the second of which summarized discrepancies in chromosome numbers in several species, impelled the writer to investigate this cytologically favorable organism.

MATERIALS AND METHODS

Samples of water containing the organism were inoculated into sterile soil-water tubes (Pringsheim, 1946) and tubes containing an inorganic solution (Bold, 1949) fortified with 5% supernatant from soil-water medium.

Twenty clonal cultures were started by introducing single cells in sterile soil-water tubes. In the soil-water tubes and inorganic salt medium with soil-water supernatant, the growth of the organism was never as good as that observed in the natural habitat. Only a very delicate green phototactic ring appeared at the surface of the culture solutions. A sample of water from the barrel in which the organisms occurred was filtered several times and analyzed for salt content. The latter was found to be approximately 0.022 N in NaCl after titrating for the Cl^- ion by the Mohr method. Sea water from the coast of Texas taken from the Port Aransas area proved to be approximately 0.66 N in NaCl. To obtain the same number of moles of NaCl for a culture solution, as present in the original habitat, 33 ml. of the Gulf sea water were diluted to one liter by adding inorganic medium and soil-water supernatant. After considerable experimentation excellent growth was obtained in a medium of the following composition:

inorganic medium (Bold, 1949)	917 ml.
soil-water supernatant	50 ml.
Gulf sea water	33 ml.

This medium supported good growth both in the liquid state and when solidified with agar.

The addition of sea water to the medium, although not essential for growth, proved very stimulatory to the algal cultures. It was also of interest to note that six of the more vigorous clones would grow in media with much higher concentrations of sea water.

---

[1] Investigation initiated while the author was Assistant in the Marine Botany Course, summer of 1958. The author wishes to acknowledge gratefully the friendly help and suggestions given by Dr. Harold C. Bold in the development of this paper.

[2] The same organism was present in these barrels as long ago as 1948 and seems to recur every year.

With this evidence that the organism grows best in relatively high concentrations of salts, as compared with that in the more commonly employed algal culture media, a modified Knop's solution was compounded as follows:

10% $Ca(NO_3)_2$	10 ml.	Gulf sea water	33 ml.
5% KNO_3	5 ml.	(approx. 4.3% salts by weight)	
5% $MgSO_4 \cdot 7H_2O$	5 ml.	Sterile rain water	892 ml.
5% KH_2PO_4	5 ml.	Soil-water supernatant	50 ml.

This solution has a salt concentration more than five times that of Bold's (1949) inorganic medium. This solution, with the sea water substituted for 33 ml. of sterile water, contains approximately 0.32% salts by weight, and was employed as the culture medium throughout the remainder of the observations. One clonal culture was inoculated into four sets of tubes containing the modified Knop's

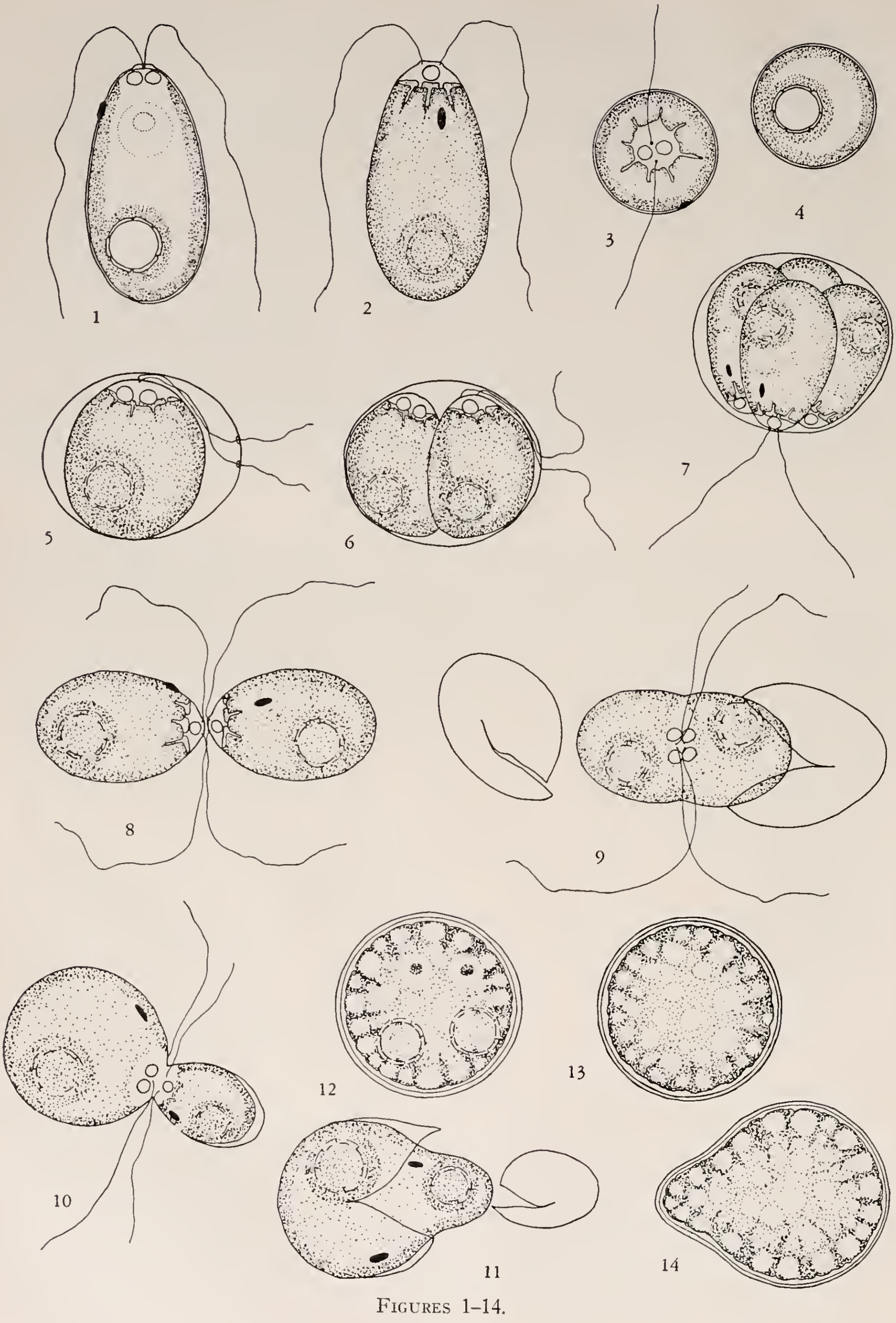
Table I

Growth of Chlamydomonas microhalophila after two weeks in Knop's medium with 5% soil-water supernatant and varying concentrations of Gulf sea water. Approximate salt content shown in parentheses

Concentration of sea water and salt content	Clone H-1	Clone H-2	Clone H-3	Clone H-4
4.3% (.36%)	++*	++	++	++
5.3% (.40%)	++	++	++	++
6.3% (.45%)	+	++	++	++
7.3% (.49%)	++	+	++	++
8.3% (.53%)	+	+	++	+
9.3% (.57%)	+	+	+	+
10.3% (.62%)	+−	+−	+−	+−
11.3% (.66%)	+−	+−	+−	+−
12.3% (.70%)	+−	+−	+−	+−
13.3% (.75%)	+−	+−	+−	+−
14.3% (.79%)	+−	+−	+−	+−
15.3% (.83%)	+−	+−	+−	+−
16.3% (.88%)	+−	+−	+−	+−
17.3% (.92%)	+−	+−	+−	+−
18.3% (.96%)	+−	+−	+−	+−
19.3% (1.00%)	+−	+−	+−	+−
20.3% (1.04%)	+−	−	−	+−
21.3% (1.09%)	+−	−	−	−
22.3% (1.13%)	−	−	−	−
23.3% (1.18%)	−	−	−	−

* Explanation of the symbols:

++	abundant growth (cultures dark-green),
+	moderate growth,
+−	scant growth,
−	no growth apparent macroscopically.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Figures 1–14.

solution with various concentrations of Gulf sea water. Scant growth occurred in the solutions containing as much as 1.0% salts (Table I).

Hanging-drop preparations were used for observing living cells. All cultures were kept under constant fluorescent illumination at an intensity of 600 to 800 foot-candles and at a temperature of 15–17° C. Flagella were observed by staining motile cells with fixative described below, modified by increasing its iodine content to the point of saturation.

The following cytological methods were employed. Motile cells were taken from the surface of densely-populated stock cultures growing in liquid media and spread over the surface of sterile agar media in Petri dishes. These were illuminated at an intensity of 800 foot-candles for about six hours. The cultures were then observed, from time to time, under a stereoscopic binocular microscope for evidences of cell division. The maximum number of dividing cells and nuclear division figures was obtained at approximately 10 to 12 hours. Cells were fixed to glass slides and these were placed in Coplin jars containing fixative, as described by Buffaloe (1958). The writer also used the fixative which Cave and Pocock (1951) had modified from Johansen (1940), except that he reduced the iodine from 5 to 2.5 grams. The stained chromosomes were seen better when the starch granules were not as heavily stained, as in the case when a more concentrated iodine fixative is used. Slides were allowed to remain in the fixative for approximately three hours, and then were drained of excess fluid. The preparations were then flooded with aceto-carmine, prepared according to the method of Cave and Pocock (1951), and placed upon a hot plate with the thermostat set at 300° F. In a very short time vapors arose from the stain. After 1½–2 minutes steaming, during which the stain turned a deep red color, the slides were removed from the hot plate, drained and destained in 45% acetic acid for approximately 10 seconds. The slides next were placed in a mixture of equal volumes of 45% acetic acid and 95% alcohol for two minutes, and, then, 95% alcohol for 5 minutes. Finally, after the alcohol bath, a drop of Euparal was placed upon the area occupied by the fixed cells and covered with a cover glass.

All figures were drawn with the aid of a Spencer camera lucida, and reduced ½ in reproduction. The magnifications are: for Figures 1–4 and 15–18, 2000 ×; for Figures 5–9 and 19–24, 1750 ×; for Figures 10–14, 1500 ×. All figures were drawn from living material except Figure 12, which was stained with I_2-KI solution, and Figures 19–24 which were stained with aceto-carmine.

FIGURES 1–14. *Chlamydomonas microhalophila.* FIGURE 1, vegetative cell in median longitudinal optical section, showing nucleus, pyrenoid, stigma and form of chloroplast. FIGURE 2, vegetative cell in surface view. FIGURE 3, vegetative cell in anterior polar view showing the incised apex of the chloroplast and the relationship of the contractile vacuoles to the plane of attachment of the flagella. FIGURE 4, vegetative cell in transverse optical section at the level of the pyrenoid. FIGURES 5–7, asexual reproduction; in this case, parental flagella functional during division. FIGURE 5, note 90° rotation, protoplast rotated 90° from longitudinal axis of cell wall; cell is still motile due to incomplete withdrawal of flagella. FIGURE 6, first cleavage completed. FIGURE 7, second cleavage completed, with the four daughter cells rotated in line with the longitudinal axis of mother cell wall. FIGURES 8–18, sexual reproduction. FIGURE 8, gametes after initial entanglement of flagella which are now completely separated; gametes probably attached by a protoplasmic thread. FIGURE 9, fusion in progress; flagella still slightly motile; note discarded gamete walls. FIGURE 10, pseudoheterogamous pair. FIGURE 11, the same, somewhat later. FIGURE 12, zygote stained with I_2-KI, showing two distinct nuclei and pyrenoids 48 hours after plasmogamy. FIGURES 13, 14, dormant zygotes.

OBSERVATIONS

Morphology and reproduction

The organism is ellipsoidal. The anterior and posterior poles are broadly rounded, but the anterior one is more acuminate than the posterior. Cell size ranges from 8.5 μ–20 μ in length and 5 μ–12 μ in width with the population averaging 14 μ in length and 8.5 μ in width. Young cells from germinating zygospores may be as small as 6 μ in length and 3 μ in width. The variation in cell size is a reflection of phases of development after liberation of daughter cells from the parent cell walls. The papilla, which is most clearly visible in small, young cells, is truncate, and it becomes obscured with increase in cell size and wall thickness (Figs. 1 and 2). Two small flagellar orifices may be observed when the protoplast contracts from the wall during cell division (Fig. 5).

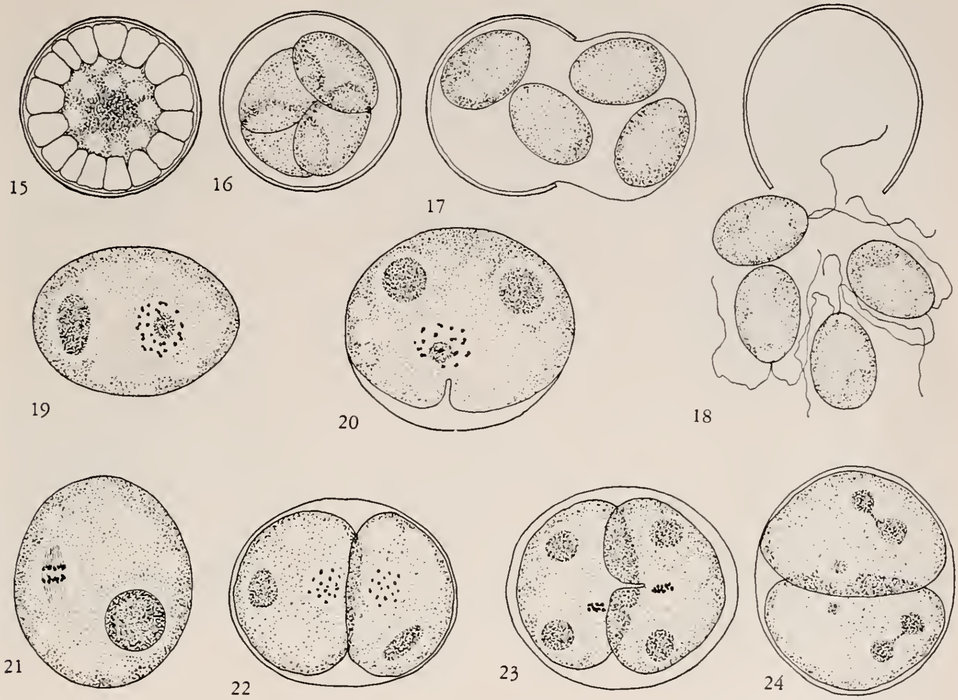
The chloroplast in median, optical section is a relatively thick-walled, hollow, ovoidal structure open at the anterior end. Here the chloroplast displays an irregularly scalloped margin (Figs. 2 and 3). The inner surface of the plastid is slightly undulate. The single spherical pyrenoid always lies in a lateral position, in a thickening of the chloroplast, in the posterior third of the cell (Fig. 1). The elliptical, disc-shaped stigma is embedded in the periphery of the anterior third of the chloroplast. The cell wall protrudes slightly at the region of the stigma (Fig. 1). The size and form of the stigma were constant in all cells observed.

The nucleus is anterior in the colorless cytoplasm. Both in living and stained cells a large nucleolus is visible, but a centrosome could not be demonstrated even with Heidenhain's iron haematoxylin, as reported for *C. terricola*. Two contractile vacuoles occupy the anterior portion of the protoplast and always lie in a plane perpendicular to that of the attachment of the flagella (Figs. 3 and 25). The latter are considerably longer than the cell. Flagella nearly twice the cell length are very common among the smaller cells.

The presence of zygotes in the twenty clonal cultures proved the organism is homothallic. The gametes are not distinguishable from the vegetative cells, except for their smaller size, an indication that, as in most species of *Chlamydomonas,* only young cells are sexually active. The gametes are isogamous; although fusion between gametes of unlike size was observed, this is explained by the fact that they are in various stages of maturation (Figs. 10 and 11).

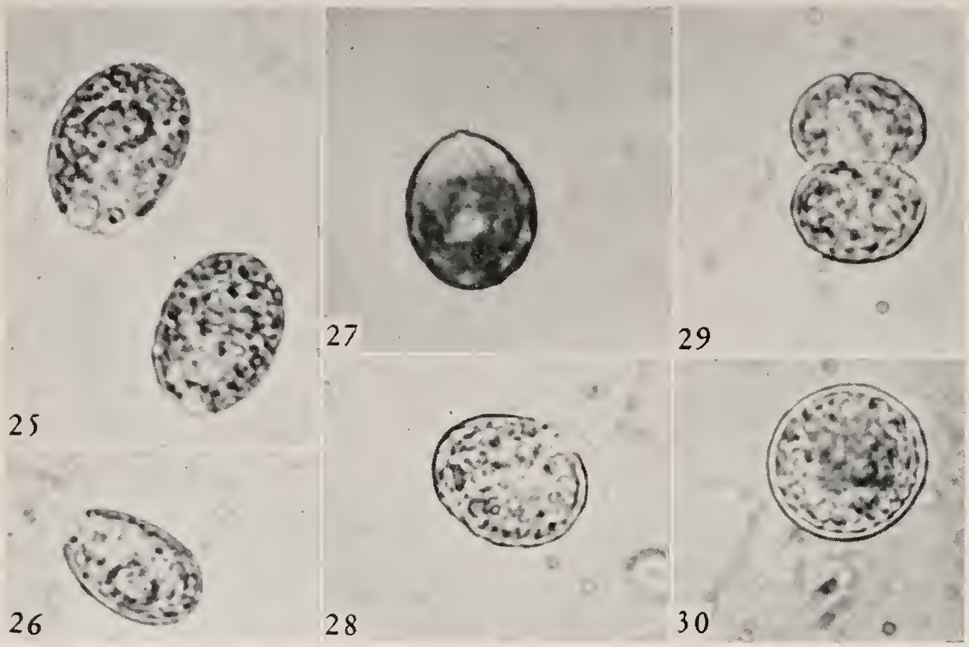
In sexual union two gametes, with free flagella, repeatedly and vigorously approach each other in the region of the papillae as observed by Bold (1949) in *C. chlamydogama*. After one to two hours of this behavior, they become almost motionless. During this process the pairs do not move very far from a given point, another similarity to *C. chlamydogama*. A protoplasmic thread, as described by Bold (1949) and Lewin and Meinhart (1953), probably unites the two cells at this stage, but because of the constant movement of the cells this thread was not observed with certainty. The space between the papillae and four free-moving flagella is very good evidence that this thread does exist in this species (Fig. 8).

After two to three hours, a permanent union of the gametes occurs at the region of the papillae, as the flagella continue to beat very feebly. The gamete walls are shed (Fig. 9) as in *C. chlamydogama*. The union of the gamete protoplasts is very slow, sometimes covering a period of 5 to 6 hours. The flagella disappear and within 24 hours a zygote is formed. If the gametes are of equal size a spherical zygote results (Fig. 13); if the gametes are of unequal size, a "pear-shaped" zygote is formed, resulting possibly from the denser consistency of the larger cell (Fig. 14). A thick wall is secreted around the zygote. Both pyrenoids and nuclei are visible in the zygotes for as long as 48 hours (Fig. 12). In some instances, the pyrenoids were visible after several weeks. The zygote enlarges to as much as 22 μ in diameter as it matures. Dormant zygotes several weeks old accumulate droplets of colorless oil in the periphery, with the chlorophyll concentrated in the center (Figs. 15 and 30). With increasing age the oil droplets enlarge. Very large, reddish-orange colored oil droplets, as confirmed by Sudan III, were observed in dormant zygotes 6 months old.



FIGURES 15–24. *Chlamydomonas microhalophila.* FIGURE 15, six-month-old zygote in median optical section with large oil droplets concentrated at the periphery. FIGURES 16–18, zygote germination. FIGURE 19, early prophase of nuclear division showing some of the chromatin bodies before condensation into chromosomes, pyrenoid elongating. FIGURE 20, pyrenoid has divided and cytokinesis is being initiated in the region of the nucleus; the latter in late prophase showing 16 chromosomes. FIGURE 21, anaphase stage with approximately 16 chromosomes moving toward the opposite poles; the pyrenoid has not divided nor has cytokinesis been initiated. FIGURE 22, first cleavage is completed, and nuclei are in prophase stage for second division; pyrenoid in one of the cells is elongating. FIGURE 23, second cleavage, pyrenoids divided, and chromosomes at metaphase. FIGURE 24, division of pyrenoids preceding second cleavage.

To effect germination, zygotes which had dried on agar, under illumination of 800 foot-candles for a period of over six months, were flooded with distilled water and kept in darkness for three days. After this, a small volume of soil-water supernatant was added and the tubes illuminated. The first germination occurred within 48 hours. At the end of six days most of the zygotes had liberated four small, motile daughter cells approximately 6 μ in length (Figs. 16, 17 and 18).



FIGURES 25–30. *Chlamydomonas microhalophila.* Photomicrographs of living cells, except Figure 27. FIGURE 25, mature cell just prior to division; note contractile vacuoles. FIGURE 26, immature cell, median optical section; note unilateral pyrenoid and chromatophore thickness. FIGURE 27, cell treated with I_2-KI, showing flagella. FIGURE 28, rotation of protoplast prior to cleavage. FIGURE 29, beginning of second cleavage. FIGURE 30, maturing zygote.

Cytology

As in many species of *Chlamydomonas*, the protoplast rotates 90° within the wall prior to cell division. Cytokinesis in this species is initiated by the appearance of a furrow in the region of the nucleus, and opposite the position of the pyrenoids or dividing pyrenoid (Fig. 20). The first division occurs perpendicular to the longitudinal axis of the cell. This coincides with the description given by Kater (1929) for *C. nasuta,* Akins (1941) for *Carteria crucifera,* Bold (1949) for *C. chlamydogama,* and by Buffaloe (1958) for four species of *Chlamydomonas.*

The two daughter cells usually undergo one more division which takes place in the same manner (Figs. 23 and 29). Cytokinesis is unilateral in the cytoplasm surrounding the nucleus (Figs. 20 and 23). Buffaloe (1958) reported that for four species of *Chlamydomonas* he studied, there was no exact synchrony between the division of the nucleus and the division of the pyrenoid. The same is true for the present organism, but there is synchrony between division of the pyrenoid and cytokinesis. It was observed many times that cytokinesis is initiated while the nucleus is still in the prophase stage (Fig. 20). The division of the pyrenoid appears always to signal the inception of cytokinesis. The division of the pyrenoid follows its elongation (Figs. 19 and 24). Nuclear division may begin before or after the initiation of cytokinesis (Figs. 20 and 21).

Each dividing mother cell usually gives rise to two or four daughter cells (Figs. 7, 22 and 23). Sometimes 8 and in exceptional cases 16 and even 32 cells were observed. Cell division generally takes place in non-motile cells, the flagella of which have been withdrawn. Occasionally, the mother cell does not become entirely non-motile, and following one or two successive bipartitions, two or four daughter cells propelled by the original flagella may be observed swimming about slowly and in cumbersome fashion (Figs. 5, 6 and 7).

The interphase nucleus is approximately 4 μ in diameter and possesses one large nucleolus. During early prophase 30 or more irregularly shaped, darkly-stained bodies may be observed, scattered about the nucleolus (Fig. 19). In later prophases, the nucleolus disappears and the darkly-stained chromosomes number approximately 16 (Fig. 22). These 16 spherical and slightly oblong bodies were never observed to form a ring in the metaphase as reported by Buffaloe (1958) for *C. reinhardti*. The polar view of the metaphase stage appears rather as a solid disc consisting of irregularly scattered chromosomes. Fewer than 16 ± 1 chromosomes were never observed. Early anaphase stages with spindle fibers clearly visible also exhibited two sets of approximately 16 chromosomes moving toward opposite poles (Fig. 21).

Discussion

On the basis of the morphological and cytological data reported in this paper, the writer attempted to ascertain the specific identity of the *Chlamydomonas* studied by consulting the literature. The organism is somewhat suggestive of *C. terricola* Gerloff (1940) but differs from it clearly in a number of respects such as position of the stigma, nuclear position, chromosome number, behavior of gamete walls at copulation and nature of the zygote wall, among others. It differs from *C. intermedia* Klebs in the anterior position of the nucleus and posterior position of the pyrenoid. Further search of the literature has failed to reveal an organism with a combination of attributes like those of the organism studied in the investigation here reported. Therefore, it is described as a new taxon, *C. microhalophila* sp. nov., the specific name an allusion to its tolerance of a relatively high concentration of salt, as compared to other species. The specific diagnosis follows:

Chlamydomonas microhalophila [3]

Cellulae ellipsoideae, ad polum anteriorem paululum attenuatae; magnitudo cellularum, secundum aetatem, 8.5–20 μ long. atque 5–12 μ lat. Chromatophorus cavus urceolatus, paululum infra polum anteriorem abrupte terminatus atque incisus, incrassatione unilaterali prope basim, pyrenoideum prominens continente, praeditus. Stigma anterius protuberans; nucleus anterior. Duae vacuolae pulsantes atque duo flagella longitudine corpori aequa aut longiora. Numerus chromatosomatum (n) = 16. Planta homothallica, in reproductione sexuali isogamica, membranis gametarum tempore coniunctionis sexualis, zygotum efferentis, omnino adiectis. Zygota matura usque ad 22 μ diam., membranan levem habentia, in quattuor cellulas filias plerumque germinantia.

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[3] The writer is grateful to Dr. Hannah T. Croasdale for preparing the Latin diagnosis.

Origo: In doliis magnis aquae plenis in loco Supply Department dock, M.B.L., Woods Hole, Mass. dicto.

SUMMARY

1. Morphological and cytological observations of a microhalophilic alga are described and illustrated.

2. Clonal cultures isolated from a barrel of water at the Marine Biological Laboratory, Woods Hole, Massachusetts, proved to be members of an undescribed species of *Chlamydomonas*.

3. The organism is described as *C. microhalophila* sp. nov., a member of the *Chlamydella* section of the genus.

4. The organism tolerates concentrations of salts (predominantly NaCl) up to approximately 1.0%, and responds by marked increase in growth in concentrations up to approximately 0.5%.

5. Its homothallic sexual reproduction and zygote germination are described and figured.

6. Its chromosome number has been determined as n = 16 ± 1.

7. Cultures of the organism have been deposited in the Culture Collection of Algae, Department of Botany, Indiana University, and herbarium specimens have been sent to the Chicago Natural History Museum.

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THE PRESENCE OF FERTILIZIN HAPTENS WITHIN THE UNFERTILIZED SEA URCHIN EGG[1]

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Tyler and Brookbank (1956a) have shown that rabbit antisera against purified fertilizin are capable of reacting with the hyaline layer of fertilized eggs, and of inhibiting the mitotic division of these eggs. This indicates a similarity between the hyaline layer combining groups or haptens and fertilizin haptens. Absorption of anti-fertilizin sera with sperm or coelomic fluid ("blood") does not remove the reaction of anti-fertilizin sera with fertilizin or hyaline layer material (Tyler and Brookbank, 1956b), indicating that species antigens are not involved. In addition, antisera against extracts of jelly-free unfertilized and fertilized eggs also possess properties of antisera against fertilizin (Tyler and Brookbank, 1956a). The possibility therefore exists that fertilizin haptens may be present within the eggs. The present report is concerned with the presence of fertilizin haptens within a granular fraction of the unfertilized egg.

Materials and Methods

Fertilizins of *Strongylocentrotus purpuratus* (Friday Harbor, Washington) and *Lytechinus variegatus* (Sea Horse Key, Florida) were prepared from acid- (pH 3.5) treated unfertilized eggs by the method of Tyler (1949). After a pre-injection control bleeding, rabbits were injected on alternate days, over a three-week period, with ca. 50 μg of fertilizin. Two intravenous injections alternated with a single intraperitoneal injection. The sera were recovered 4–5 days after the final injection, and thoroughly dialyzed against sea water. Reaction of the sera with fertilized eggs (cleavage block), fertilizin (ring precipitin test), and sperm (agglutination) was recorded.

Preparations of adenosine-triphosphatase-bearing granules (ATPase-granules) were made according to a method devised by Whiteley (unpublished data). Unfertilized eggs were deprived of soluble fertilizin by acid (pH 3.5) treatment, and thoroughly washed. Since acid-treated eggs are fertilizable, and are agglutinated by solutions of antifertilizin, it is apparent that some fertilizin remains on the surface after acid treatment. In order to reduce possible contamination of the ATPase-granules with this remaining fertilizin, the eggs were treated for 20

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[1] This investigation was supported in part by a research grant (RG 4659) from the National Institutes of Health of the Public Health Service.

[2] The author wishes to express his gratitude to the Friday Harbor Laboratories of the University of Washington for the use of space and equipment during the summer of 1957, to Professor A. H. Whiteley, of the University of Washington, for permission to include some of his unpublished results in this report, and to Dr. Ruth Cooper, for a critical reading of the original manuscript.

minutes with 10 mg.% trypsin in sea water (crystalline, lyophillized trypsin, Worthington Biochemical Company, Freehold, New Jersey) prior to homogenization. Eggs so treated were found to have reduced (no greater than 25% cleavage in 0.25% sperm suspension) fertilizability as compared with controls (ca. 90% cleavage in 0.25% sperm suspension), and also a reduced capacity to absorb antibodies against fertilizin (Table I). In order to minimize the amount of fertilizin present on the surface, the eggs were, therefore, routinely trypsinized in this manner prior to homogenization. One ml. of the washed, settled eggs was homogenized with 9 ml. of cold KCl-citrate solution (1 part .35 *M* Na citrate: 9 parts .55 *M* KCl, pH 6.8). Following two low speed centrifugations to remove unbroken eggs etc., the ATPase-granules were recovered, as a yellow pellet, by two successive centrifugations of 15 minutes duration (10° C.) at 10,000 × gravity. The resuspended particles were spherical, of uniform size (ca. 2 microns in diameter) and readily distinguishable from the larger yolk granules which remain, for the most part, in the supernatant. Such small-granule preparations have 85% of the total ATPase activity of the whole egg homogenate (Whiteley, unpublished data). Preparations of this sort, with no more than an estimated 10% contamination by yolk granules, were used as absorbing antigens (preparations which contained little or no discernible yolk were also successfully utilized). Prior to use in absorptions, the yellow pellets were rinsed with sea water to remove the KCl-citrate mixture.

TABLE I

The effect of trypsin treatment on the capacity of unfertilized eggs to absorb antibodies against fertilizin

	Serum	Ring with fertilizin	
#10	(pre-injection serum)	±	
10′	(antiserum) unabsorbed	+++	
10′	trypsinized egg absorbed	+++	2nd absorption +
10′	egg absorbed (no trypsin)	+	2nd absorption ±

Sperm which were used as absorbing antigens were centrifuged and washed three times to remove seminal fluid. "Blood" was obtained from KCl-injected adult animals through a puncture in the peristomial membrane, and examined for contamination with eggs or sperm. This material was then allowed to clot at room temperature. The clot was recovered by centrifugation, washed with sea water, and used as an absorbing antigen. Jelly-free unfertilized eggs, deprived of the soluble portion of their fertilizin coat by acid treatment (pH 3.5), were also used for absorptions. For each experiment, approximately equal volumes of serum and absorbing material were mixed for 5 minutes at room temperature, and then centrifuged to recover the serum.

Clear supernatants of homogenized blood clots and homogenized ATPase-granule supernatant were used as test antigens in ring precipitin tests, as were fertilizin solutions. Visible precipitin reactions occurring within 2–5 minutes were scored as +++, while those appearing after 30 minutes are indicated as +. Reactions appearing after 90 minutes are indicated by ±. Tests were carried out with undiluted sera in tubes of ca. 1.5 mm. internal diameter.

Tests for cleavage blocking activity involved mixing one drop of egg suspension (about 100 eggs) and one drop undiluted serum (sea water dialyzed). The sera were scored as blocking (+++) if no more than one cell division of the membraneless fertilized eggs occurred following the addition of the serum. Retardations of development without inhibition of cleavage are indicated by a plus-minus sign (±).

RESULTS AND DISCUSSION

The results of experiments with the two species of sea urchin are summarized in Table II. From this table it can be concluded that absorption with eggs or ATPase-granules removes most of the cleavage-blocking activity of the immune sera, as well as the majority of the fertilizin precipitins; sperm absorption is not effective, nor is absorption with blood. Absorption with blood removes virtually all antibodies against this material, though sperm absorption leaves antibodies, capable of precipitating soluble blood antigens, in solution. Since the unabsorbed Lytechinus antisera against fertilizin do not react with sperm, it is reasonable that sperm absorption should fail to abolish any reactions exhibited by these sera. The reaction of *L. variegatus* anti-fertilizin sera with ATPase-granule supernatant may be due to the presence of unsedimented ATPase-granules in this material, or to the presence of fertilizin haptens associated with some other cytoplasmic fraction.

Concerning contamination of the ATPase-granules with surface fertilizin, all that can be said, at present, is that attempts have been made to hold this to a minimum. As can be seen in Table I, the trypsinized eggs show only a *reduction* in ability to absorb antibodies against fertilizin, indicating that some fertilizin remains after this treatment (further trypsin treatment tends to make the eggs excessively fragile, and therefore difficult to handle). Quantitative studies on the amount of granular material necessary to absorb a given volume of antiserum are necessary before the effectiveness of trypsin treatment in reducing possible contamination of the ATPase-granules can be evaluated. On the other hand, the acid-treated eggs were washed three times with sea water, and, following trypsin treatment, an additional five times with sea water and three times with the homogenization medium. It is therefore unlikely that any soluble fertilizin is available, for adsorption to internal constituents, at the onset of homogenization. Small particles of fertilizin, in an insoluble complex of some sort, may be present and able to combine with ATPase-granules or other fractions.

These results are of value in interpreting the similarity of action of antisera against purified fertilizin and antisera against extracts of "jelly-free" unfertilized and fertilized eggs. Both types of antisera block cleavage, precipitate fertilizin, and may fail to agglutinate sperm (Tyler and Brookbank, 1956a, 1956b). In addition, antisera against Lytechinus fertilizin, and against "jelly-free" unfertilized and fertilized Lytechinus eggs, increase the respiration rate of unfertilized and fertilized eggs (Tyler and Brookbank 1956b; Brookbank, 1959).

The presence of fertilizin haptens within the eggs may be of importance in the chemical "architecture" of the eggs. Tyler (1940) discovered that unfertilized sea urchin eggs contain a substance complementary to the surface fertilizin (termed antifertilizin from eggs). This discovery and other investigations (Tyler, 1946) led Tyler (1947) to propose an auto-antibody concept of cell structure and cell adhesion, involving a system of interlocking, mutually complementary substances extending from sites of synthesis to the external boundary of the cell. The demonstration of fertilizin haptens within the eggs would be consistent with this

TABLE II

Ring tests, sperm agglutination, and cleavage inhibition tests on antisera against fertilizin

	Ring with fertilizin	Agglutination of sperm	Cleavage block	Ring with "blood"	Ring with ATPase granule supernatant
S. purpuratus					
Pre-injection sera					
d. unabsorbed	o	o	o		
d. sperm absorbed	o	o	o		
d. ATPase-granule absorbed	o	o	o		
e. unabsorbed	±	o	o		
e. sperm absorbed	±	o	o		
f. unabsorbed	o	o	o		
f. sperm absorbed	o	o	o		
Immune sera					
d. unabsorbed	+++	+	+++		
d. sperm absorbed	+++	o	+++		
d. ATPase-granule absorbed	o	+	±		
e. unabsorbed	+++	o	+++		
e. sperm absorbed	+++	o	+++		
f. unabsorbed	+++	±	+++		
f. sperm absorbed	+++	o	+++		
L. variegatus					
Pre-injection sera					
#4. unabsorbed	±	o	o	o	o
#4. sperm absorbed	±	o	o	—	—
#4. blood absorbed	o	o	—	o	—
#4. unfert. egg absorbed	±	—	o	—	o
#4. ATPase-granule absorbed	o	—	o	—	o
#10. unabsorbed	±	o	o	o	—
#10. sperm absorbed	±	o	o	o	—
#10. unfert. egg absorbed	o	—	o	—	—
#10. ATPase-granule absorbed	±	—	o	—	—
Immune sera					
4. unabsorbed	+++	o	+++	+	+
4. sperm absorbed	+++	o	+++	+	—
4. blood absorbed	+++	o	+++	±	—
4. unfert. egg absorbed	±	—	o	—	±
4. ATPase-granule absorbed	±	—	o	—	o
10. unabsorbed	+++	o	+++	+	—
10. sperm absorbed	+++	o	+++	+	—
10. unfert. egg absorbed	±	—	o	—	—
10. ATPase-granule absorbed	+	o	±	—	—

— indicates test not performed.

theory. However, the presence of fertilizin haptens, as demonstrated by serological techniques, does not, of course, necessarily imply that the substances bearing these haptens are able to combine specifically with antifertilizin of eggs of sperm. Or, to rephrase the foregoing sentence, there is no reason to assume that the rabbit antibodies against fertilizin are directed, exclusively or in part, against the fertilizin-antifertilizin combining sites.

SUMMARY

1. ATPase-bearing granules of unfertilized sea urchin eggs were shown, through absorptions of antisera against fertilizin, to possess fertilizin-like combining groups.

2. These granules were also capable of neutralizing the cleavage-blocking action of these antisera.

3. These results are discussed in light of the similarity of action of antisera against purified fertilizin to the action of antisera against extracts of jelly-free (acid treated) unfertilized eggs, and washed, demembranated fertilized eggs.

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X-RAY EFFECTS ON MITOTIC ACTIVITY OF THE ACCESSORY SEX ORGANS OF CASTRATE RATS STIMULATED BY TESTOSTERONE PROPIONATE[1]

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The effects of irradiation of the mouse testis with 320 r of x-rays have been discussed in a previous paper (Bryan and Gowen, 1956). The testis is characterized by a relatively high level of mitosis and in this respect is quite different from most other organs. Our findings indicate that irradiation markedly inhibited mitotic activity in spermatogonia. In addition data were obtained which suggested that irradiation-induced inhibition of desoxyribose nucleic acid (DNA) synthesis contributed to this suppression of mitotic activity. Observations on other tissue cells would broaden the significance of these findings both to the normal mitotic behavior of these cells and to the effects of radiation on them.

The mitotic behavior of the accessory organs of the castrate rat, the seminal vesicle and the dorsal prostate, is of significance to this problem. These organs may have high mitotic rates. They have the further advantage that the rates may be controlled through castration which reduces mitotic activity through removal of hormonal stimulation and/or by testosterone injections which enhance the mitotic activity (Moore *et al.,* 1930; Burrows, 1940; Cavazos and Melampy, 1954; Melampy *et al.,* 1956 and others).

The response of these tissues to irradiation and/or hormone treatments as described herein was measured by mitotic counts coupled with cytophotometric measurements of the DNA-Feulgen content of interphase nuclei. This approach has the advantage that the methods complement one another. Together they provide information with respect to the relations between the visual manifestations of mitotic activity and certain underlying biochemical activities.

Materials and Methods

Male rats of Fischer line 344 were used in the present experiments. The animals were castrated when nine weeks old and the accessory organs allowed to regress for twenty days. All the experimental animals were then given daily subcutaneous injections of testosterone propionate (500 μg) in oil.[2] Half the animals were anesthetized and exposed to 320 r of x-rays (130 pkv; 10 ma; filtration 0.25 mm. Al; anode-target distance 20 cm. in air; dose rate 320 r/min.). The irradiation was delivered to the pelvic region only, the rest of the body being shielded with lead. The irradiation was given coincident with the initial hormone injection.

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[2] Peranderan, Ciba Pharmaceutical Products, Inc., Summit, N. J.

Pairs of animals were killed at 24, 48, 60 and 72 hours following irradiation and/or initial hormone injection. The seminal vesicles and dorsal prostate were rapidly removed, cut into small pieces, blotted to remove any secretion and dropped into the fixative. Tissues were fixed overnight in 10% neutral formalin, washed for 24 hours in running water and then divided into two portions. One was dehydrated, cleared in benzene and embedded in 56–58° C. Tissuemat; the other was taken up to 70% alcohol and stored in the refrigerator. Kidney tissue from control rats also was fixed and processed in the above manner.

Table I

The effects of irradiation and of androgen injection on the mitotic activity of the secretory epithelium of the accessory organs of castrate male rats

Treatment	Seminal vesicle			Dorsal prostate		
	No. cells counted	% Mitosis	Mitosis as % of control	No. cells counted	% Mitosis	Mitosis as % of control
Intact controls	7,697	0.25	—	7,733	0.52	—
20 day castrates	8,753	0.10	40	8,861	0.06	11.5
20 day castrates:						
(a) 24 hrs. after 1st hormone injection	9,280	0.38	152	7,326	0.38	73.1
24 hrs. after 1st hormone injection and x-rays	7,637	0.41	164	6,796	0.43	82.7
(b) 48 hrs. after 1st hormone injection	7,575	1.49	596	8.171	0.71	136.5
48 hrs. after 1st hormone injection and x-rays	13,375	1.30	520	8,752	0.63	121.1
(c) 60 hrs. after 1st hormone injection	7,162	2.76	1,104	11,901	2.05	394.2
60 hrs. after 1st hormone injection and x-rays	7,832	3.12	1,248	7,570	0.99	190.4
(d) 72 hrs. after 1st hormone injection	9,901	3.53	1,412	11,742	1.97	378.8
72 hrs. after 1st hormone injection and x-rays	7,254	2.25	900	7,603	1.82	350.0

The embedded material was sectioned at 6 μ and the slides therefrom were used for mitotic index determinations. The material stored in alcohol was used to prepare isolated nuclei for photometric purposes since examination of sectioned material indicated that most nuclei were badly overlapped.

Small pieces of tissue were run down to water and hydrolyzed in 1 N HCl for 12 minutes at 60° C., washed, and stained *in toto* by means of the Feulgen reaction for two hours at room temperature (Stowell, 1945). After passing through the bleach baths the tissue pieces were washed in distilled water, passed into 45% acetic acid and left for 10 minutes. Small fragments were then removed into

TABLE II

An analysis of the variation in the mitotic index data

Treatment	Animals compared	Between organs or rats			Among counts			Binomial error	
		M.S.	df	F[1]	M.S.	df	F		df
Control	Rat A—S.V. & D.P.	.000	1		.004	2		.003	6903
	Rat B—S.V. & D.P.	.042	1	8.9**	.002	4		.005	8458
	Rats A & B—S.V.	.000	1		.003	4		.002	7673
	Rats A & B—D.P.	.029	1		.003	3		.005	7688
20-day castrate	Rat A—S.V. & D.P.	.001	1		.000	1		.001	7503
	Rat B—S.V. & D.P.	.000	1		.000	2		.001	10,090
	Rats A & B—S.V.	.000	1		.000	2		.001	8740
	Rats A & B—D.P.	.000	1		.000	1		.001	8853
24-hr. hormone	Rat A—S.V. & D.P.	.001	1		.001	5		.004	8851
	Rat B—S.V. & D.P.	.001	1		.004	4		.003	7707
	Rats A & B—S.V.	.000	1		.003	5		.004	9238
	Rats A & B—D.P.	.003	1		.002	4		.004	7320
24-hr. hormone + x-rays	Rat A—S.V. & D.P.	.000	1		.002	3		.004	7121
	Rat B—S.V. & D.P.	.001	1		.001	3		.004	7242
	Rats A & B—S.V.	.001	1		.001	4		.004	7600
	Rats A & B—D.P.	.000	1		.003	2		.004	6963
48-hr. hormone	Rat A—S.V. & D.P.	.040	1	4.4*	.009	3		.009	7054
	Rat B—S.V. & D. P.	.281	1	23.4**	.003	4		.012	8510
	Rats A & B—S.V.	.118	1	7.9**	.006	4		.015	7450
	Rats A & B—D.P.	.002	1		.006	3		.007	8108
48-hr. hormone + x-rays	Rat A—S.V. & D.P.	.005	1		.008	7		.007	11,591
	Rat B—S.V. & D.P.	.348	1	24.8**	.016	6		.014	10,290
	Rats A & B—S.V.	.404	1	30.3**	.014	8		.013	13,191
	Rats A & B—D.P.	.000	1		.008	5		.006	8690
60-hr. hormone	Rat A—S.V. & D.P.	.010	1		.097	4	4.0**	.024	9304
	Rat B—S.V. & D.P.	.358	1	15.5**	.054	5	2.4*	.023	9304
	Rats A & B—S.V.	.044	1		.093	3	3.4*	.026	6959
	Rats A & B—D.P.	.084	1	4.2*	.063	6	3.1**	.020	11,649
60-hr. hormone + x-rays	Rat A—S.V. & D.P.	1.191	1	56.7**	.025	4		.021	7617
	Rat B—S.V. & D.P.	.689	1	34.4**	.009	4		.020	7454
	Rats A & B—S.V.	.027	1		.029	4		.031	7582
	Rats A & B—D.P.	.007	1		.005	4		.010	7489
72-hr. hormone	Rat A—S.V. & D.P.	1.190	1	37.0**	.064	6	2.0	.032	11,593
	Rat B—S.V. & D.P.	.185	1	9.4**	.009	5		.020	9454
	Rats A & B—S.V.	.744	1	21.2**	.074	5	2.1	.035	9544
	Rats A & B—D.P.	.136	1	6.8*	.010	6		.020	11,503
72-hr. hormone + x-rays	Rat A—S.V. & D.P.	.072	1	3.5	.061	4	3.0*	.021	7246
	Rat B—S.V. & D.P.	.011	1		.081	4	4.0**	.020	7298
	Rats A & B—S.V.	0.012	1		.061	4	2.7	.022	7085
	Rats A & B—D.P.	.003	1		.081	4	4.5**	.018	7459

[1] F values marked * shows significance at the 0.05 level and ** at the 0.01 level.

drops of 45% acetic acid on microscope slides, covered with a coverslip and macerated with the aid of a Burgess Vibro-graver equipped with a plastic tip. By this means epithelial nuclei were isolated in a manner suitable for photometric purposes.

TABLE III

DNA-Feulgen content of seminal vesicle and dorsal prostate nuclei

	Seminal vesicle			Dorsal prostate		
	Class	N	DNA-Feulgen content	Class	N	DNA-Feulgen content
Intact controls	II	50	3.58 ± 0.07	II IIa	49 1	3.26 ± 0.06 4.41
20-day castrates	II	50	3.13 ± 0.06	II III	49 1	3.11 ± 0.07 6.36
20-day castrates: (a) 24 hrs. after 1st hormone injection	II	50	2.92 ± 0.06	II IIa III	48 1 1	2.35 ± 0.08 3.51 4.27
24 hrs. after 1st hromone injection and x-rays	II IIa	49 1	2.58 ± 0.07 4.60	II	50	2.49 ± 0.06
(b) 48 hrs. after 1st hormone injection	II IIa III	25 7 18	3.05 ± 0.10 4.35 5.73 ± 0.12	II IIa III	40 2 8	2.74 ± 0.07 4.13 5.71
48 hrs. after 1st hormone injection and x-rays	II IIa III	38 5 7	3.14 ± 0.08 4.80 6.05	II IIa III	43 3 4	2.74 ± 0.08 4.29 5.35
(c) 60 hrs. after 1st hormone injection	II IIa III	25 9 15	2.61 ± 0.11 3.83 4.96 ± 0.27	II IIa III	37 5 8	1.63 ± 0.06 2.65 3.19
60 hrs. after 1st hormone injection and x-rays	II IIa III	27 6 17	2.61 ± 0.11 4.18 4.74 ± 0.11	II III	48 2	2.10 ± 0.08 3.87
(d) 72 hrs. after 1st hormone injection	II IIa III	39 2 9	3.11 ± 0.08 4.42 5.88	II	50	2.39 ± 0.06
72 hrs. after 1st hormone injection and x-rays	II IIa III	26 14 10	2.74 ± 0.11 4.19 ± 0.10 5.73 ± 0.46	II IIa	49 1	2.24 ± 0.04 3.67

The slides were frozen on a block of dry ice and the coverslips removed. Slides were passed into 95% alcohol, further dehydrated and mounted in oil of matching refractive index.

Measurements of the DNA-Feulgen complex were made with the apparatus as described previously (Bryan and Gowen, 1956). Transmittance data were ob-

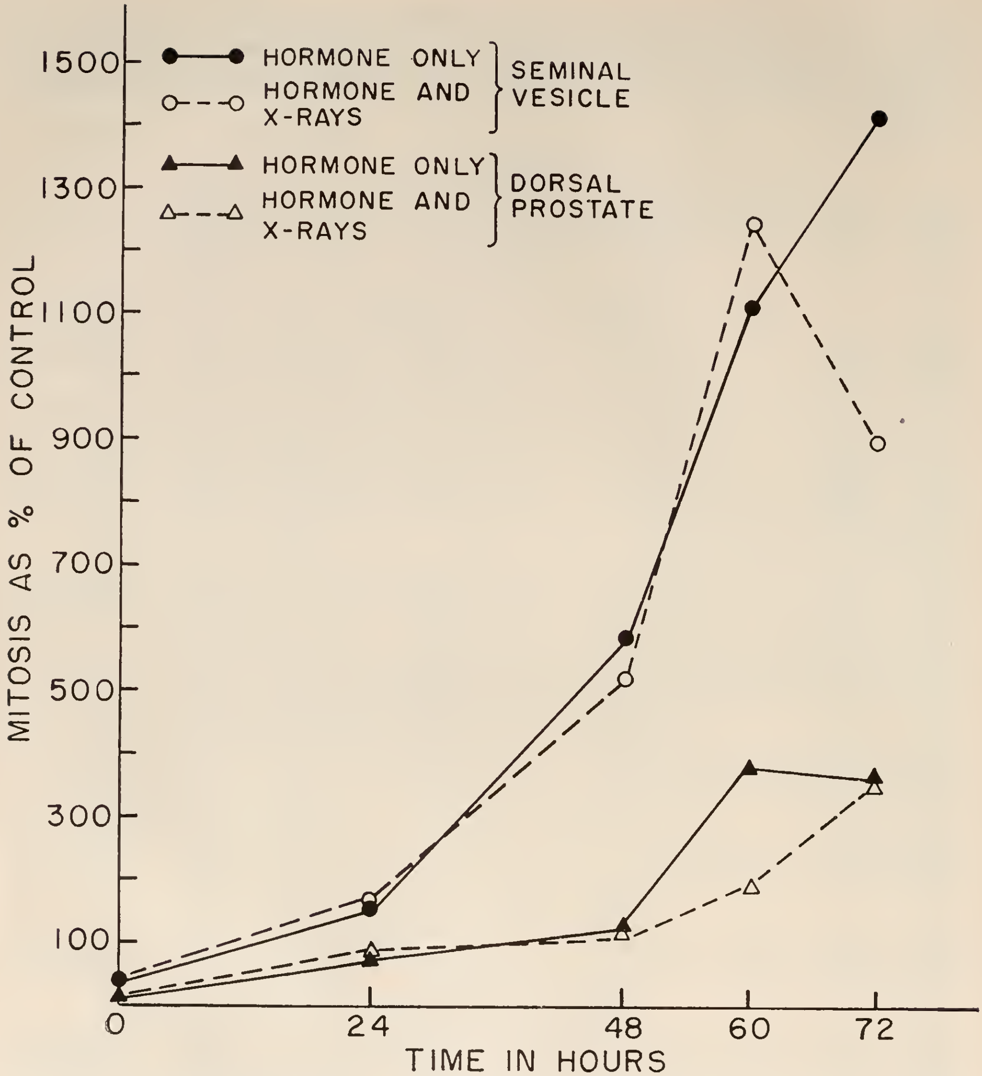

FIGURE 1. The mitotic response of seminal vesicle and dorsal prostate epithelium at different times following irradiation and/or hormonal stimulation.

tained by the "plug" method of Swift (1950); however, since the majority of nuclei were ellipsoidal rather than spherical, the formula derived by Kasten (1956) was substituted for the one devised by Swift.

An important factor in the utilization of the Feulgen procedure for cytophotometric purposes is the precise control of the hydrolysis step. When small pieces of tissue—rather than thin sections—are used, the hydrolysis procedure becomes subject to more variation. The end result is that DNA-Feulgen values for the same tissue processed in two separate procedures may be more variable than the results obtained with sectioned material processed in a similar manner. However, for any single piece of tissue the staining appears to be quite uniform. Thus in the case of the kidney, DNA-Feulgen measurements made on fragments drawn from different regions of the same piece did not differ significantly from each other. This experiment was repeated at a later date with a fresh batch of Feulgen reagent and similar results were obtained. However, the mean values for the DNA-Feulgen content were about 28% higher. In view of these findings and of the fact that the slides were made at different times involving different batches of the Feulgen reagent, care must be taken not to misinterpret the variation between the mean DNA-Feulgen values reported in Table III.

Feulgen-stained sections were used for mitotic index determinations. A total of 3000–6000 cells was counted and classified per tissue per animal. Fields were chosen at random, every cell in the field being classified and recorded.

RESULTS

A. *Mitotic index determinations*

The data obtained are summarized in Table I. The values in columns 2, 3, 6 and 7 of the table represent the combined counts from each pair of animals. In columns 5 and 9 the mitotic activity is expressed as per cent of control values for seminal vesicle and dorsal prostate, respectively. These values are presented in graphical form in Figure 1.

Seminal vesicle

The data indicate that by 20 days following castration the level of mitotic activity has declined to about 40% of the control value. The hormone-treated animals show a steady increase in activity reaching a peak at 72 hours after the initial injection was given. The level of activity reached at this time represents a fourteen-fold increase over the value obtained for the controls. In the case of the animals receiving irradiation as well as hormone, the rise in mitotic activity is similar over the period 0 through 48 hours. Here, however, the peak of mitotic activity is reached at 60 hours and the level then undergoes a marked decline. This 60-hour value is not very different from the corresponding value of the animals which received hormone alone.

Dorsal prostate

In this organ the response was found to be quite different from that of the seminal vesicle (Fig. 1). At no time during the experiment did the levels of mitotic activity of the dorsal prostate reach levels comparable with those determined for the epithelium of the seminal vesicle. The level of activity increased more slowly over the period 0 through 48 hours. In the case of the animals which received hormone alone the mitotic activity reached a peak at 60 hours and essentially the same level was found at 72 hours. Unlike the conditions prevailing in the seminal vesicle, the mitotic activity in irradiated and hormone-treated animals did not reach a peak until the end of the experimental period, the value being almost identical with the terminal value for the hormone-only group.

In certain pairs of animals some variation in the mitotic index is apparent. As examples of the magnitude of these variations the most extreme cases are presented herewith. In the seminal vesicle, the 48-hour hormone + x-ray data range from 0.75% (rat A) to 1.83% (rat B). Similarly in the case of the dorsal prostate, mitotic counts for the 72-hour hormone animals range from 1.63% to 2.29%. A statistical analysis of the variation in the mitotic index data is set forth in Table II. The variation between seminal vesicles at 48 and 72 hours following the initial hormone injection and at 48 hours after hormone and irradiation is significant at the 1% level. The dorsal prostates show some variation between rats at 60 and 72 hours after the start of the hormone treatment. These differences in response are significant at the 5% level.

On account of this observed variation between animals in the present work, differences in the magnitude of the mitotic response of irradiated versus unirradiated animals should be viewed with circumspection. However, the data do establish the trend in response to the treatments. Within this framework, the mitotic counts when taken together with the DNA-Feulgen data do allow meaningful conclusions to be drawn.

B. *DNA-Feulgen content of nuclei*

The data obtained are presented in Table III. Tissue from each member of pairs of animals was subjected to the photometric procedure. From each animal and tissue, samples of twenty-five nuclei were measured. The means are therefore based on samples of fifty measurements each. The mean amount of DNA-Feulgen complex, in arbitrary units, together with their respective standard errors are listed in columns 4 and 7 of the table. Nuclei possessing the diploid amount of DNA-Feulgen complex are designated as Class II nuclei, those with twice this amount Class III, and Class IIa represents nuclei possessing an amount of DNA-Feulgen complex intermediate between these levels.

The range of values for the DNA classes was determined from measurements of nuclei isolated from kidney tissue of control animals. The spread of values was slightly variable; thus two samples gave highest values of 1.4–1.5 units higher than the lowest (for example, ranging from 2.69 units to 4.17 units) while in two samples measured at a later date a range of 1.2 units was obtained (2.15–3.37 units).

Since the kidney nuclei have a more uniform appearance than the seminal vesicle or dorsal prostate nuclei, the larger range of values was chosen as a better approximation to the range expected for the diploid class. An approximation to the range expected for Class III nuclei was made by doubling the values obtained for the Class II nuclei in each set of measurements. Any values falling between the upper limits of Class II and the lower limits of Class III were assigned to Class IIa.

Seminal vesicle

It is evident from Table III that until 48 hours following irradiation and/or the initial hormone injection, the DNA-Feulgen values fall almost entirely into Class II. At 48 hours significant numbers of Class III nuclei appear but they are much less frequent in the irradiated material. By 72 hours following the initial hormone injection the frequency of Class III nuclei has declined to about half the 48-hour value. In the case of the irradiated animals the highest number of Class III nuclei appears in the 60-hour material.

This variation in number or lack of Class III and Class IIa nuclei is not due primarily to errors of sampling (though the latter may contribute to the variation) since duplicate samples have yielded essentially the same results.

Dorsal prostate

In the section on mitotic activity it was pointed out that the epithelium of the dorsal prostate was much less mitotically active than that of the seminal vesicle. This behavior is paralleled by the synthetic activity—as judged by the lower frequency of Class IIa and Class III nuclei. Not only are these classes almost absent until the 48-hour period, but also at 72 hours following the start of the hormone treatment. This latter decline is in contrast with the findings with respect to the seminal vesicle.

Discussion

As pointed out earlier, the testis is characterized by a high rate of spermatogonial mitosis. It is this mitotic activity which is markedly inhibited following irradiation. Other data indicate that inhibition of DNA synthesis in interphasic spermatogonial nuclei contributes to this suppression of mitotic activity. It would therefore seem likely that other mitotically active tissues would respond in a similar manner following exposure to similar x-ray doses.

A brief resumé of the spermatogonial response is given here to facilitate comparison with the results of the present work. In the normal mouse testis 4 per cent of the spermatogonia appeared to be undergoing mitosis at a given time. Spermatogonial divisions rapidly declined to less than 5 per cent of the control level by 3 days after exposure to 320 r of x-rays. A rise in mitotic activity was apparent 5–10 days following irradiation. The highest level of activity was not observed until the termination of the experiment, *i.e.,* at 28 days after exposure. At this time the level attained was slightly more than twice that of the controls.

As may be seen from Table I and Figure 1, the mitotic response of the accessory organs follows a pattern quite different from that just described. The onset of activity occurs in a matter of hours rather than days. This suggests that the period of mitotic inhibition is markedly contracted.

In the case of the seminal vesicle peak mitotic activity is reached at 60 hours after irradiation. Moreover, this peak value is approximately 12 times higher than the control level. The mitotic response of this organ is·then much stronger and more rapid than in the case of the testis.

With respect to the dorsal prostate the highest level of activity following irradiation was not reached until 72 hours after exposure. This level was 3.5 times higher than the control. The response of the dorsal prostate is clearly on a much lower level than that of the seminal vesicle.

It would appear from the above discussion that the radiation response of somatic and germinal tissue is quite different. This may be a reflection of underlying metabolic differences between tissues. This idea receives some support from the recent work of Pelc and Howard (1956). These workers, using C^{14}-labelled adenine, found a difference in incorporation between spermatogonia and somatic tissues such as seminal vesicle, skin and intestine. Their data suggest that a DNA precursor becomes maximally labelled soon after injection and is drawn upon by spermatogonia but not by somatic tissues. There is then the possibility that the DNA of reproductive cells may be synthesized in a somewhat different manner from that of somatic cells. These biochemical processes may differ in their sensitivity to x-rays.

Certain other factors may also contribute to the observed differences in response. As is evident from our present data and from the prior work of Cavazos and Melampy (1954), hormonal stimulation of mitotic activity does not, in these organs, become markedly effective until 48 hours after the initial injection. It has been pointed out by Lea (1955) that the effect of the x-ray dose decays with time (see page 289). So it is possible that the effect of the dose has undergone some decay before the inhibitory action can become effective. In other words, the "effective" dose of x-rays may not be identical for the two experiments.

The response of the seminal vesicle to androgen and irradiation treatments has been studied by several other workers (see for example Cavazos and Melampy, 1954; Fleischmann and Nimaroff, 1954; Melampy *et al.,* 1956). The results reported here differ in some aspects from those of the studies; however, taken as a whole they do show similar trends. These previous workers used colchicine to facilitate mitotic index determinations whereas colchicine was not used in the experiments reported here. Therefore the mitotic indices listed in Table I are much lower than those published by the workers cited above.

The data of Fleischmann and Nimaroff (1954) and of Melampy *et al.* (1956) were interpreted as suggesting that following irradiation there is a delay in the onset of mitotic activity. After doses of 640 r or less (Melampy *et al.,* 1956) the delay was such that the peak level of mitotic activity was observed at 60 hours. Furthermore, Fleischmann and Nimaroff (1954) found that if an interval of 5 days was allowed between irradiation and hormone injection the delaying effect was lost. This latter observation is of interest inasmuch as it clearly demonstrates that recovery occurs after a partial-body exposure to 3000 r.

In the present work, the 60-hour mitotic index following exposure to 320 r was only about 12% higher than that of the controls (hormone alone) whereas Melampy *et al.* found corresponding values to differ by a factor of two. Strain differences may be involved since the Fischer strain (line 344) appears to be more radioresistant than certain other strains (Dunning, personal communication). On this basis a shortening of the delay period would be expected with the consequence that the mitotic index would be depressed towards control levels. Also from a statistical viewpoint, the numbers of animals per time period are rather small so that errors of sampling are of increased significance (see elsewhere in this paper for further discussion of sources of variation which bear on this point).

As shown in Figure 1, the mitotic response of the dorsal prostate following irradiation and/or hormonal stimulation is on a much lower level than that of the seminal vesicle. This is most probably a reflection of innate differences in activity of these organs as well as possible differences in magnitude of the response to the experimental treatments. The shape of the curves for the dorsal prostate would suggest that recovery from any induced mitotic delay does not occur until close to the end of the experimental period. This is in contrast with the findings for the seminal vesicle.

It has been shown by various workers that the synthesis of chromosomal material in preparation for mitotic division occurs during the interphase. Experiments with radio-active precursors by Pelc and Howard (1952), Taylor and McMaster (1954) and recently, by Taylor *et al.* (1957), point clearly in this direction. Cytophotometric studies (see for example Swift, 1950; Bryan, 1951; Taylor and McMaster, 1954) are in agreement with the results obtained by autoradiographic procedures.

From the above work it should follow that any treatment which suppresses or inhibits mitotic activity should result in the absence or reduction in number of Class III nuclei. In a sample of nuclei selected at random from a mitotically active tissue the relative proportions of the DNA classes will depend upon the speed at which DNA synthesis is accomplished and the rate at which Class III nuclei enter into prophase. Thus if conditions are such that Class III nuclei enter division without any intervening delay, the chances of encountering such nuclei in a sample are rather small. If, on the other hand, chromosomal reduplication is proceeding at a rate appreciably faster than that at which such nuclei enter division, then Class III nuclei should accumulate and should constitute a significant proportion of the measured sample. The mitotic activity—measured in terms of reduplication of the DNA content of interphase nuclei—will then be a measure of the balance between these mechanisms. It should also follow that, within certain limits, a comparison of the DNA-Feulgen data and the mitotic counts should afford some insight with respect to the nature of the response evoked by agents which inhibit or enhance mitotic activity.

In the case of the intact controls, where mitotic activity was found to be rather low and most probably represented replacement of dead cells rather than tissue growth, 98–100% of the nuclei measured fell into the diploid DNA class (Class II). Similar results were obtained with respect to the 20-day castrates where the mitotic index was still lower. Not until the 48-hour period were significant numbers of Class III (and IIa) nuclei encountered. With respect to the seminal vesicle material, the proportion of Class III nuclei at this time was 63% in the case of the unirradiated animals, whereas following irradiation 14% of the sample was composed of such nuclei. At 60 hours after the initial hormone injection the proportion of Class III nuclei remained about the same. In the case of the 60-hour irradiation and hormone-treated material, the proportion of Class III nuclei had increased to 34% (from 14% at 48 hours). At 72 hours after the start of the treatment, the proportion of Class III nuclei had fallen to 18% of the sample from animals receiving hormone alone and to 20% in the case of the irradiated animals.

These cytophotometric data indicate that the tissue response evoked by the irradiation treatment is rather similar to the response to hormone injection but that a difference in timing is apparent. In the case of the animals receiving only hormone, the highest frequency of Class III nuclei is reached at 48 hours following the initial injection. After irradiation, on the other hand, the peak frequency is not attained until 60 hours after exposure. Moreover, following irradiation, the

60- and 72-hour frequencies are rather similar to the corresponding values for animals receiving hormone alone.

It should be recalled that the frequency of Class III nuclei most probably represents a balance between the rate of chromosomal reduplication and the rate at which nuclei enter into prophase of mitosis. From this it would follow that the rise in frequency of Class III nuclei very probably is an indication of increased synthesis following hormonal stimulation. By the same token, the slower rise in frequency noted in the irradiated material suggests that in such material the synthetic rate responds less rapidly to the hormonal stimulus. The present data then may be interpreted to mean that irradiation does interfere with the synthetic mechanism of these hormonally stimulated tissues.

The data pertaining to the dorsal prostate showed little change from the control level until the 48-hour period. At this time the frequencies of Class III nuclei following irradiation and/or hormone were 8% and 16%, respectively. At 60 hours, the frequency of such nuclei in the sample from irradiated material had fallen to 4%, while the corresponding value for unirradiated material remained unchanged from the 48-hour level. At the termination of the experimental period (72 hours) the samples measured contained no Class III nuclei regardless of treatment.

These cytophotometric data show that the response of the dorsal prostate is on a much lower level than that determined for the seminal vesicle. Just as in the case of the mitotic counts, the DNA-Feulgen data point up a response to the experimental treatments which is different from that shown by the seminal vesicle.

As is the case with the mitotic counts, the DNA-Feulgen data show that the response of these somatic tissues to irradiation and/or hormonal stimulation is much more rapid than in the case of the spermatogonia. In the latter the frequency of Class III nuclei reaches its highest level (51%) at about 10 days following irradiation and thereafter declines rapidly to about 5% of the sample at 28 days. These data when considered along with the corresponding mitotic counts indicate a rapid utilization of Class III nuclei during the regenerative period. From the present DNA-Feulgen data it may be seen again that a situation analogous to the spermatogonial one presents itself but over a much shorter period of time.

The mitotic index determinations and the DNA-Feulgen data taken together allow the interpretation that hormonal stimulation of mitotic activity does not, in these organs, become markedly effective until 48 hours after the initial injection. Furthermore, the data prior to the 48-hour period lend themselves to the conclusion that mitotic activity is very closely correlated with the rate of chromosomal reduplication (formation of Class III nuclei). It would also appear that by 48 hours the rate of chromosomal reduplication is beginning to run ahead of mitotic activity and, with respect to the seminal vesicle, that this "imbalance" is maintained to a lesser degree for the duration of the experimental period. With respect to the dorsal prostate similar conclusions may be drawn with the notable distinction that over the period of 48 through 72 hours the "balance" has again changed in a manner such that, at the termination of the experiment, the close correlation evident earlier has been restored.

The results reported here lead to the conclusion that exposure to a similar dose (320 r) of x-rays is much less effective in inhibition of mitosis in these tissues (under conditions of hormonal stimulation) than in the case of the spermatogonia. The DNA-Feulgen data may be interpreted to mean that, following irradiation, DNA synthesis suffers some impairment but recovers in a relatively short period of time. The demonstrated mitogenic action of testosterone propionate may have in some manner interfered with, or masked, the inhibiting action of the irradiation. Such an idea receives some support from the work of Rugh and Clugston (1954). These authors found that the stage of the oestrus cycle (at the time of irradiation) affected the radiation sensitivity of female mice. They found that females in dioestrus were about twice as sensitive (in terms of the male LD 50/30 days—625 r) to the irradiation as were females in oestrus. Thus an increased level of oestrogen appears to be correlated with increased resistance to x-irradiation. The same may be true also for androgens, especially so since both classes of steroid hormones elicit similar responses in experimental animals (see Bullough, 1952).

The present results, together with our earlier data (mouse testis), not only imply that the male germ line may differ from somatic tissues in its response to irradiation, but also that somatic tissues may differ from one another in this respect. At the present time it would appear that differences between the tissues studied are mainly ones of degree and rate of response. However, the findings of Pelc and Howard (1956) point out the possibility that somatic and germinal tissues may also differ in their metabolic pathways. Until more biochemical and cytological information is at hand the precise nature of these differences must remain an open question.

Summary

1. The accessory organs of castrated male rats have been used in a study of the effects of x-rays on mitosis in somatic tissues.

2. Animals nine weeks old were castrated and the organs allowed to regress for 20 days prior to use. Half the animals received daily injections of 500 μg of testosterone propionate (in oil) until death; the others, in addition to the hormone injections, were exposed to a single dose of 320 r of x-rays, the irradiation being given at the time of the first hormone injection. Pairs of animals were killed at 24, 48, 60 and 72 hours following the irradiation and/or initial hormone treatment.

3. The response to the treatments was followed by means of mitotic index determinations and cytophotometric measurements of the DNA-Feulgen content of interphase nuclei.

4. The cytological data indicate the existence of a difference in response between the epithelium of the seminal vesicle and of the dorsal prostate. At no time during the experiment did the mitotic activity of the latter rise to levels characteristic of the former. In addition, the time-response curves for the two organs indicate that the dorsal prostate responds more slowly than the seminal vesicle.

5. The DNA-Feulgen measurements together with the mitotic index data indicate that in the controls and in the experimental animals killed prior to 48 hours there is a close correspondence between the level of mitotic activity and the rate of chromosomal reduplication. Over the period of 48–72 hours in the case of the dorsal prostate the data show that, during the time of maximal hormonal stimulation, DNA synthesis is proceeding at a rate appreciably faster than the rate at which nuclei enter into prophase.

6. The results obtained have been compared with those obtained from a similar experiment involving the mouse testis. The accessory organs appear to be less sensitive to the irradiation than the testis. Factors bearing on this point are discussed.

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ANNUAL REPRODUCTIVE CYCLES OF THE CHITONS, KATHERINA TUNICATA AND MOPALIA HINDSII[1]

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The reproductive season of marine invertebrates has been determined in a variety of ways: spawning, the appearance of young in plankton, increase in size of gonad relative to the body, and development of ripe gametes (see Giese, 1959, for reference). In most cases an annual reproductive season has been observed, but many species breed more than once during the season, some showing a monthly rhythm (Korringa, 1947). Data on reproductive seasons of marine invertebrates are still not extensive and the problems posed are numerous. It is desirable that data on many more species be gathered to gain a better perspective. This paper records observations on reproduction in two species of chitons, *Katherina tunicata,* an intertidal form, and *Mopalia hindsii,* a pile dweller.

METHODS

Ten specimens of *Katherina* were gathered monthly from the mid-littoral zone of Carmel Point during 1954–55 and from the same zone at Yankee Point during 1956–58, in each case from rocks in the *Postelsia* and mussel zone exposed at low tide. The specimens at Carmel Point were larger but the population was sparser, and perhaps less representative than that at Yankee Point. The sample from Yankee Point was therefore selective inasmuch as very small specimens were avoided (weights: 1957, 16–44 gms.; 1958, 11–53 gms.)

Ten specimens of *Mopalia* were obtained during monthly low tides from the pilings at Monterey harbor. The population in this region is unusual in that the specimens are preponderantly large and not too numerous, therefore the samples may not be truly representative of the species (weights: 1957, 14–73 gms.; 1958, 16–65 gms.). However, this species was not found in other accessible environments in sufficient numbers for sampling.

Each chiton was weighed, the foot removed and the animal eviscerated, permitting easy removal of the gonad. The gonad volume was determined and the gonad index (the volume of the gonad is divided by the weight of the animal and multiplied by 100) determined.

To determine whether any striking changes in the content of nutrients occurred in the blood during the course of the reproductive cycle of *Katherina,* the reducing sugar, protein and non-protein nitrogen were determined monthly in samples of blood. For this purpose the blood of from ten to twenty individuals

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[1] Supported in part by U.S.P.H. Grant 4578 to A. C. Giese. We are indebted to Mrs. M. Lusignan, Mr. G. Araki, Mr. J. Bennett, Mr. A. Farmanfarmaian, Dr. A. T. Giese and Mr. John Schmaelzle for cartographic, statistical and sampling help.

[2] Now at the Department of Zoology, University of California at Los Angeles.

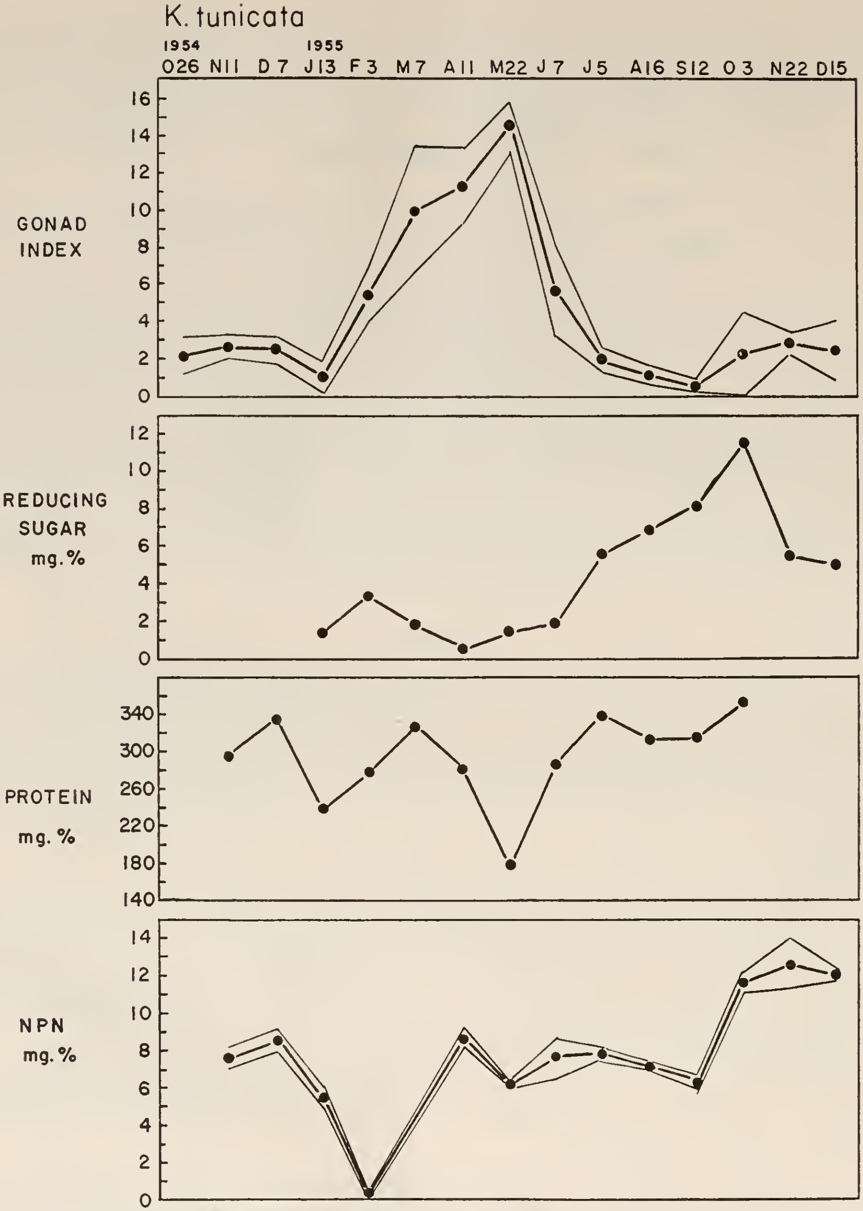

Figure 1. Top: Reproductive cycle of *Katherina tunicata* for 1955 as determined by the gonad index. The gonad index is the ratio of gonad volume to wet weight of the entire animal times 100. The specimens were collected at Carmel Point, California. The dots indicate the means and the lines, the 95 per cent confidence limits. Lower three graphs show variation in reducing sugar, protein and non-protein nitrogen in the population samples used for gonad analyses.

had to be pooled because a single animal does not have enough for analysis, a 33-gram animal giving only several milliliters of blood. Blood samples were obtained from the lateral sinuses following puncture through the mantle cavity on either side of the animal. The blood is a yellowish fluid which was not observed to coagulate although it showed some murkiness on standing, suggesting a feeble clumping of the blood corpuscles.

Reducing sugar was determined by the anthrone method (Seifter *et al.,* 1950) after precipitating the proteins with 10 per cent trichloracetic acid. Non-protein nitrogen was determined with the micro-Kjeldahl after precipitating the protein, and total nitrogen was determined by the same methods, without the preliminary addition of protein-precipitating agents. Protein was taken to be the difference between these two volumes multiplied by 6.25.

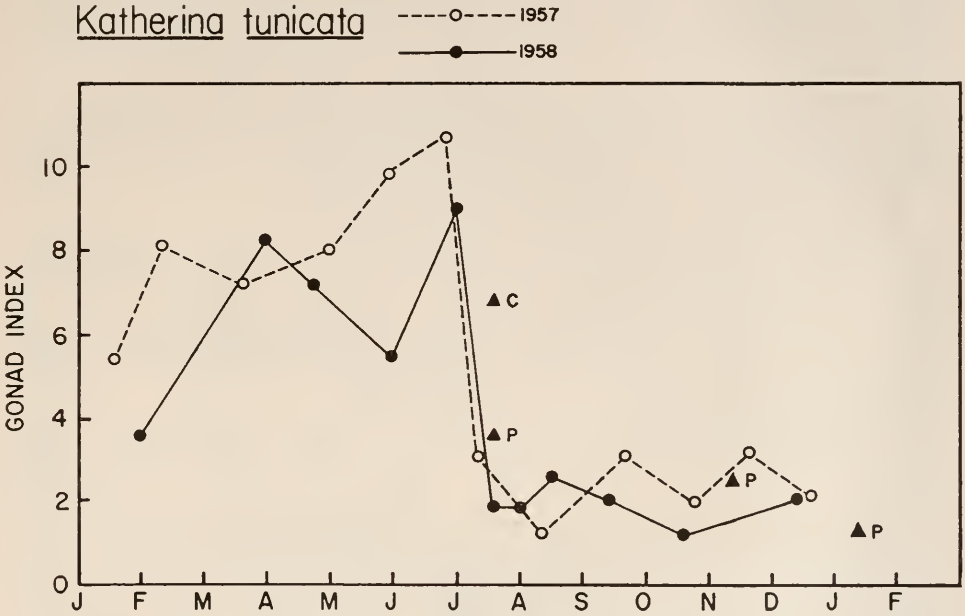

FIGURE 2. Reproductive cycle for *Katherina tunicata* during 1957 and 1958 as determined by gonad index. The specimens were collected at Yankee Point, with the exception of points marked C which came from Carmel Point and those marked P which came from Pescadero Point.

KATHERINA TUNICATA

For each of the three years during which *Katherina* was studied an annual reproductive cycle was found. It is most clear-cut for the year 1954-55 (the population collected at Carmel Point) as shown in Figure 1. It is less distinct for the collections at Yankee Point during the years 1956–57 and 1957–58, the gonad index being high for several months preceding the spawnout, as shown in Figure 2. The difference in sharpness of the cycle at the two locations may be the result of a more homogeneous population of large animals at Carmel Point. A comparison of the gonadal index for random specimens from both localities during the same year is desirable.

During the height of the season males are easily distinguished from females by the color of the gonad, which is greenish in the female (the eggs being green) and very light in the male (the sperm being white). However, it is not possible to distinguish the sexes of individuals from spawned out gonads, but the shell gland of the female is always diagnostic of sex even in a spent female. When minute spherical eggs begin to develop as a new cycle comes into prospect, diagnosis from gonads is again easy. Statistical analysis of the 1954–55 data showed no essential difference in the time of onset in maturity of testes and ovaries in males and females; therefore the data for both sexes were combined. It was, however, noted that the gonad index of males appears to be slightly higher than that of females at the height of the season and lower than that of the females during the decline of the season. The 95 per cent confidence limits for the pooled males and females given in Figure 1 indicate that the entire population of animals is relatively homogeneous, since the confidence band is never very wide.

The data for almost two years of the breeding cycle for the same population at Yankee Point, given in Figure 2, indicate that the cycle varies somewhat from year to year. After slowly spawning during the months of March, April, May and June of 1958 the gonad index again rose, suggesting minor ripples in sexual activity or a second active period because of favorable conditions in the area. During this second cycle the gonad index rose significantly from 5.5 on June 9 to 8.9 on July 9 then fell precipitously to 1.9 on July 18 of 1958.

It is interesting to note that the populations sampled at three stations on July 17 and 18 showed different average gonad indices: 6.8 at Carmel Beach, 3.6 for Pescadero Point, and 1.9 for Yankee Point. This indicates that the exact time of spawning is probably in part determined by the geographical and ecological location.

Two findings are similar for the three years of data available; 1) in all cases the chitons have small shrunken gonads late in July, and 2) the gonads remain shrunken during the late summer, fall and early winter. In the months after the beginning of winter the gonads grow rapidly and gametogenesis occurs. The reproductive cycle therefore consists of several months of stasis, several months of growth, several months during which the gonads are ready for spawning, and probably several periods of spawning. MacGinitie and MacGinitie (1949) report that *Katherina* lays eggs in the Monterey Bay area in July while Hewatt (1938) reports it spawning in the same area in May, and Rickets and Calvin (1948) say it spawns in Puget Sound a month or two earlier.

Attempts to study breeding and spawning of *Katherina* in the laboratory did not prove successful since often the animals crawled out of the tanks to dry locations, and even though periodically submersed, they deteriorated. They did not seem to eat while in the laboratory although red and brown algae were provided and a scum of diatoms was always present on the surface of the aquaria.

In its natural environment *Katherina* feeds upon diatoms and upon algae, since the pellets in the gut of animals sampled consisted of partially digested algae of various kinds, including brown and red algae. In other cases the gut was filled from end to end with diatoms and skeletons of diatoms. Apparently the chitons eat what is readily available to them and are always found within reach of algae. *Katherina* does not move out of the sunlight, remaining on the upper surfaces of the rocks during low tide. It is therefore subjected to and weathers all the changes in conditions which obtain during low tide.[3] Most chitons have tegmental aesthetes or "shell eyes" which enable them to perceive the light. These are absent in *Cryptochiton* and may be of less importance in *Katherina* in which much of the shell is covered by tissue (Crozier, 1921). A variety of other organisms are found growing upon *Katherina*. These include occasional barnacles, coralline algae, and colonial animals such as bryozoans and hydroids, all of which anchor to the skeletal plates. The overgrowth by algae and various colonial animals indicates the sessile nature of the animals and their tendency to remain on the upper surface of the rocks.

The nutrients in the blood of *Katherina* vary during the year (Fig. 1). The most prominent constituent of the blood is protein which is always present in large amounts, varying from about 180 to 240 mg. per cent. The lowest values of blood protein appear to coincide with the highest gonad index. The non-protein nitrogen of the blood is small in amount, reaching a maximal value of about 12 mg. per cent and falling to almost zero when the gonad is increasing most rapidly in size. Reducing sugar is present in rather small amounts from almost zero to about 11 mg. per cent. A single determination of the blood constituents therefore has little meaning as a value for the species since the constituents vary so much in amount from month to month. The variations in blood constituents in Figure 1 may possibly be correlated with the breeding season. Similar variations in content of organic constituents of the body fluid of echinoderms (Bennett and Giese, 1955), and in the blood of a number of species of crabs (Leone, 1953) have also been documented. It would be interesting to know whether the blood of marine invertebrates is generally so variable.

A certain amount of organic material is stored in the foot, the gonads and the hepatic gland. It is clear from preliminary tests that neither the digestive gland nor the foot has much glycogen, although almost 0.1 per cent wet weight of the soft tissue of the animal consisted of glycogen (0.83 per cent of the dry weight). Lipid seems to be more important than glycogen as a storage material. Detailed studies on these constituents are under way.

Mopalia hindsii

Mopalia hindsii is listed by MacGinitie and MacGinitie (1949) as an estuarine form which is often found on pilings. They have found some specimens as large as 10 centimeters long and 7.5 centimeters wide. The animals used in this study were usually smaller than the maximum, being generally about 8.0 centimeters long and 5.5 centimeters wide. Specimens from the pilings in Monterey Harbor were covered by a forest of bryozoans, hydroids, and a multitude of larval crustaceans, nemerteans, roundworms, annelid worms, and protozoans (including the large ciliate *Condylostoma*). This association is common to this species (MacGinitie and MacGinitie, 1949). Shells of *Mopalia* are often weakened by a boring worm (Tucker and Giese, 1959), probably of the family Spionidae.

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[3] Heath (1899, 1905) found that many of the chitons are highly sensitive to sunlight and creep into crevices, and that one species, *Ischnochiton magdalenensis,* even buries itself in the sand with the approach of day. *Katherina tunicata* and *Cryptochiton stelleri* are among the few species which remain exposed during the bright daylight. *Mopalia hindsii,* it is true, remains on the inner pilings at Monterey Harbor during the day, but this is an environment of rather attenuated light.

The intestinal contents of the specimens studied were filled with skeletons of bryozoans and other organisms which grow on the pilings which they inhabit. *Mopalia* was generally absent from the outer pilings where the light is sufficient for a relatively abundant growth of algae and were more numerous in the darker regions where bryozoans, hydroids, anemones, and starfishes occurred.

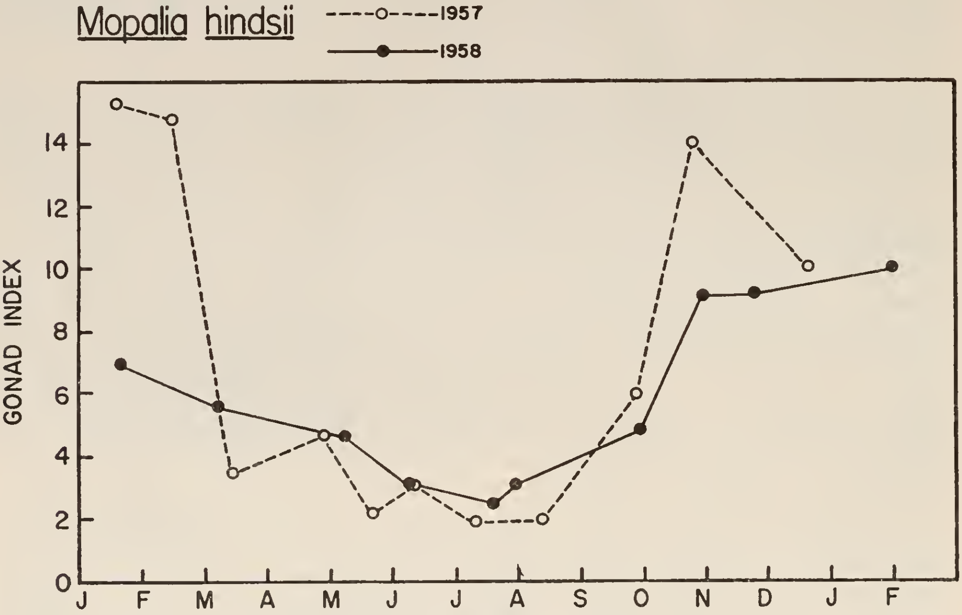

FIGURE 3. Reproductive cycle of *Mopalia hindsii* during 1957 and 1958 as determined by gonad index. Specimens collected on pilings in Monterey Harbor.

A definite annual reproductive cycle is observed for *Mopalia* (Fig. 3). Breeding, as indicated by enlargement of gonads with development of eggs and sperm, occurs in the late fall and winter, approximately from October to February or March. That it was somewhat different each year is indicated by the data in Figure 3.

DISCUSSION

A number of previous workers have described the breeding habits of various chitons (Heath, 1899, 1905; Grave, 1922; Stephenson, 1934; Costello *et al.*, 1957). Grave (1922) found that *Chaetopleura apiculata* spawns at ten-day intervals during the months of June, July and August at Woods Hole, the activity declining in the intermediate periods (for other references, see Costello *et al.*, 1957). Spawning in this species occurred during the night and is possibly associated with phases of the moon (Korringa, 1947). Stephenson (1934) found that *Acanthozostera gemmata* bred in the Great Barrier Reef of Australia from September to April, spawning occurring every four weeks during the breeding period, associated with phases of the moon. After April it entered a prolonged resting period.

Heath (1899, 1905) made many excellent observations of the breeding activities of chitons in nature. He observed *I. magdalenensis* breeding in the Monterey area in May and June, in the latter month over a considerable length of the coastline in the area. Hewatt (1938) also observed the same species breeding in June, but found *Mopalia muscosa* breeding in September. Heath (1899, 1905) found that chitons of the Monterey area do not breed in captivity.

Heath (1905) also observed that isolated males of *I. magdalenensis* and *Mopalia lignosa* spawn, but isolated females do not. However, females placed with males in a tide pool spawn soon after the males have spawned, suggesting chemical stimulation. This observation confirms similar observations by Metcalf (1892) on *Chiton squamosus* and *C. marmoratus* (see also Crozier, 1922). Heath observed shedding and egg laying in *Ischnochiton mertensii, I. cooperi, M. muscosa* and *K. tunicata* in large pools isolated by low tides. The males began shedding when the waters became tranquil after recession of the tide and stopped shedding when disturbed by water movements. Shedding sometimes continued for as long as two hours, steadily or in spurts. He bred some chitons in isolated pools and grew them to maturity. Young *K. tunicata* isolated in pools near mean tide mark grew to 25 mm. in length in one year and reached their average length of 55 mm. in three, a rapid rate of growth characteristic of other species of chitons as well.

The two species of chitons discussed in this paper, *K. tunicata* and *M. hindsii,* show a distinct breeding season which, while similar from year to year, is sufficiently distinctive each year to indicate that it is not closely tied to day-length or some other factor invariant from year to year, but rather is subject to action of various local factors. The local variation in reproductive state found in one collection at three different stations in the Monterey area (Yankee Point, Pescadero Point, and Carmel Point) during early July, 1958 indicates how important to the breeding cycle are the small differences in the ecology of the three environments.

The most curious feature of breeding in the two species of chitons studied here is the reciprocal nature of their breeding seasons, *Katherina* breeding in the summer, *Mopalia* in the winter. This resembles the pattern described for the crab *Pachygrapsus crassipes* which breeds in the summer, while *Hemigrapsus nudus,* another grapsoid crab of similar habits and nature, breeds in winter (Boolootian *et al.,* 1959). It would be valuable to ascertain what factors trigger the sweep of the reproductive cycles at a period approximately six months apart in the two species of chitons. Other cases of this type are known but in no case has an adequate experimental analysis of causal mechanism yet been made (Giese, 1959).

SUMMARY

1. The reproductive cycles of two species of chitons, *Katherina tunicata* and *Mopalia hindsii,* collected in Monterey Bay are recorded, the first for three years and the second for two.
2. *Katherina* breeds in the summer, *Mopalia* in the fall and winter.

3. In each case the gonad index (ratio of gonad volume to body weight times 100) rises gradually and falls rather precipitously as spawning occurs. Differences in onset of breeding occurred during the years for which records are available, suggesting timing of events by local conditions.

4. Blood protein, non-protein nitrogen, and reducing sugar vary during the year but it is not clear whether these variations are significantly correlated with reproductive condition.

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A STUDY OF THYROID FUNCTION IN FUNDULUS HETEROCLITUS[1, 2]

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The role of the thyroid gland in regulating the metabolic rate, and its effect on growth and other physiological functions in higher vertebrates have been well established, but in fish, despite the many studies using anti-thyroid drugs, thyroxine, thyroid extract, and thyrotropin, its function is still obscure. The diffuse nature of the thyroid gland of most teleost fishes has rendered the study of its function extremely difficult; surgical removal is impossible except in a very few species where the gland is encapsulated, and the use of anti-thyroid drugs introduces disadvantageous collateral effects (reviewed by Chambers, 1953). The availability of radioactive iodine has now made possible a new technique for the extirpation of the thyroid gland, especially suited to one of diffuse nature as found in these fish. La Roche and Leblond (1954) were the first to investigate the problem of radiation thyroidectomy in fishes. They found that total destruction of the thyroid in salmon (*Salmo salar* L.) required repeated injections of large, but progressively downgraded doses of I^{131}. Fish weighing 30–32 grams at the start of the experiment received 100, 50, 40 and 30 μC at the rate of one dose per month. Arvy, Fontaine and Gabe (1956) also used a series of injections, but only claimed to have achieved a state of hypothyroidism in rainbow trout (*Salmo gairdneri* Richardson). They gave a total dose of 260 μC in three injections at 30-day intervals, to fish weighing about fifty grams. More recently, Fromm and Reineke (1957) have reported successful destruction of the thyroid after a single injection of 250 μC to fingerling trout weighing 3.8–6 grams. In an abstract, which has not been reported in detail, Baker, Berg, Gorbman and Gordon (1955) described the effects of partial or complete thyroid destruction in platyfish by the addition of 4.5–7 μC of I^{131} to 200 ml. of aquarium water. The period of exposure was 24–48 hours, but more complete destruction resulted from the longer treatment. Fontaine, de la Querrière and Raffy (1957), in a study of the respiratory metabolism of hypophysectomized eels, noted a fall in the oxygen consumption 48 hours after the injection of 334 μC of I^{131} into fish weighing about 70 grams. This was followed by a gradual return towards normal over a period of several weeks. Olivereau (1957) has discussed the problem of dosage, tissue damage, and regeneration of the thyroid in eels treated with radioactive iodine.

In this study *Fundulus heteroclitus* was chosen as an experimental fish, not

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[2] Part of a thesis submitted to the Department of Zoology, Yale University, in partial fulfillment of the requirements for the degree of Master of Science.

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only for reasons of its hardiness and availability, but since it is a euryhaline species, it afforded the opportunity to study the possible role of the thyroid in water and salt regulation. Following an initial pilot experiment to determine the dosage of I^{131} necessary for thyroidectomy, the following experiments were designed to study the physiological effects of thyroid deficiency on growth and ability to osmoregulate, as well as effects on other organs and tissues that might possibly be dependent on thyroid function.

Materials and Methods

The fish used were *Fundulus heteroclitus* males of about 7.5 cm. in length, caught in the New Haven area in August, 1956. They were placed in storage tanks kept at 20° C. for two months before use, at which time they were measured, numbered, and selected for uniformity of weight of approximately five grams. The results of the previous experiment to determine dosage indicated that dosage levels somewhat lower than 10 μC/gm. wt. spaced over a period of several months would be the most effective procedure for destroying the active as well as the initially inactive follicles. Accordingly, in this experiment a series of graded doses was given, 25, 15, and 10 μC, spaced about five weeks apart. Thus, with five-gram fish, the doses were approximately 5, 3, and 2 μC per gram weight of fish.

On October 23, the thirty-six fish selected for I^{131} treatment were given a dose of 5 μg per fish of thyrotropin (Armour 317–51); on the following day they were given the first injection of I^{131}, 25 μC per fish in a volume of .05 ml. The injected fish were placed in five-gallon wide-mouth carboys provided with aeration and means for feeding and changing water without handling the fish, and after eight days they were returned to their regular aquaria. On November 27 the second dose of iodine was given, 15 μC per fish, with an injection of 5 μg of thyrotropin given two days before. The third iodine injection of 10 μC per fish was given on January 4, 1957, following the usual dose of thyrotropin. These fish were subsequently screened by the tracer method described below, and those showing the greatest impairment of thyroid activity were set aside for further experiments. The fish were anaesthetized with tricaine methane sulfonate (MS 222) for injections and later screening operations.

The fish were fed once daily with Aronson's formula fish food, consisting of a cooked mixture of ground beef liver, kidney, greens, dried shrimp, and Pablum. The temperature in all tanks was kept at 20° C. and illumination was ten hours per day.

Experimental setup for osmoregulation studies

To study the physiological effects of hypothyroidism following direct transfer of fish from sea water to fresh water, it is necessary to maintain tanks with sea water of constant salinity as well as several with running fresh water. A small circulating sea water system of simple design was used, with a 55-gallon polyethylene reservoir drum feeding by gravity into the aquaria, the overflow draining through a filter, and subsequently returned to the reservoir by means of a hard rubber pump. The salinity of this system was kept at 26 ‰, with a pH of 7.5 and oxygen content of 4.28 ml./liter.

A dechlorinating system for tap water similar to that described by Burden (1956) was the fresh-water source. The pH was 6.9, oxygen content 5.46 ml./liter, and a flow rate of 350–400 ml. per minute was maintained in each of the tanks.

Autopsy procedures

All fish which died during the iodine treatment or prior to the osmoregulation experiment were autopsied to determine the possible causes of death, and the thyroids were fixed in Bouin's for histological examination. At the termination of the osmoregulation experiment, the remaining fish were autopsied, measurements of standard length, weight, and testis weight were made, the thyroid was fixed in Bouin's, and blood samples were taken for hematology and chloride titration, as described below.

Hematology

Red and white cell counts, thrombocytes, and per cent hemoglobin were determined on blood samples which were allowed to drop on siliconized slides from the cut end of the tail. Heparin was used on the razor blade. The procedures will be described elsewhere by Dr. Anne M. Slicher, who made this study.

Blood chloride

Blood chloride determinations were made using a micro-adaptation of the method of Schales and Schales (see Hawk, Oser and Summerson, 1954). Whole blood was collected from tail cuts, centrifuged in an air-driven "spinning top" rotor, and 4.8-μl samples of serum were pipetted into titration vessels, closed with paraffined corks and frozen. Before titration the samples were diluted with 42.6 μl of distilled water to which indicator had been added (3 ml. stock indicator/50 ml. solution). It was found necessary to add about six drops of 2.0 N HNO_3 to the 50 ml. of diluted indicator in order to release the bound chloride in the sample.

To check the accuracy of the method, standard human serum (Hyland Laboratories, Los Angeles, lot 369E6) with known NaCl was titrated, using the same amounts and procedures as with the fish preparation. The standard contained 544 mg% with an acceptable range of 533–555 mg%. The test titrations gave a value of 549 mg%, well within the range.

Screening method for determining degree of thyroidectomy

Although all fish were ultimately examined histologically to determine the degree of thyroidectomy, it was desirable to know how effectively the thyroid had been destroyed before using the fish for experimental purposes. Thus a method using a tracer dose of I^{131} to measure the rate of activity loss in the throat region was worked out and a device was designed to hold the fish over a Geiger counter so that comparison readings could be made between normal and treated fish.

A lead block was cast around the mold of a fish to serve as shielding, as well as a holder for the experimental fish. A hole was drilled through the bottom of the block to connect with the thyroid region of the fish, and the block was mounted on a wood frame so that the opening coincided with the ¼-inch end window of a Geiger-Müller tube (Mark 1, Model 105. Radiation Counter Laboratories, Inc.,

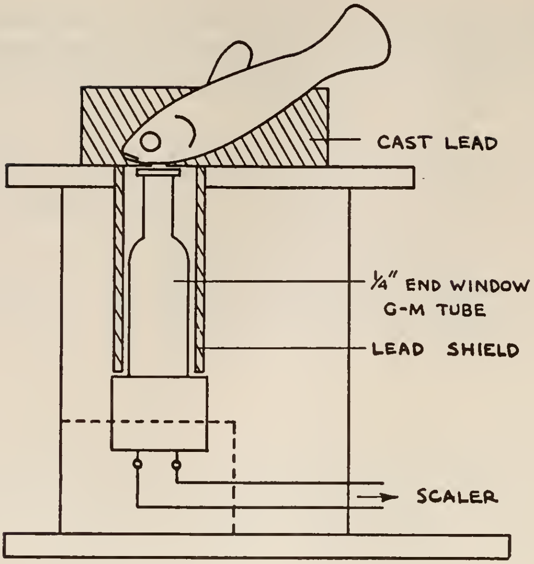

FIGURE 1. Counter arrangement for measuring activity of thyroid area.

Skokie, Ill.). Lead shielding was found to be necessary around the tube, and its photosensitivity was obviated by cementing a thin plastic film over the opening in the bottom of the block and coating with "Aquadag" (see Fig. 1).

A series of trial injections of I^{131} indicated that a dose of 2.5 μC per five-gram fish gave an adequate counting rate, and since this was also in line with tracer doses used by Gorbman and Berg (1955) and others, it was subsequently used in this screening procedure.

RESULTS

Histological studies of the thyroid

Following fixation in Bouin's solution at the time of autopsy, the thyroid regions of the experimental and control fish were examined histologically to determine the degree of thyroidectomy. Serial sections were made through the entire region, and an evaluation was made based on the relative number of follicles present, the height of the epithelial cells, and amount of colloid, in relation to the control fish. Few of the treated fish showed a total lack of follicles, but it was interesting to note that these fish, and others with very few follicles, did not survive to the end of the experiment. There was a great range in the amount of thyroid tissue present. However, while some fish showed evidence of considerable regeneration, in the majority of fish regeneration was present to a much lesser extent, and on the whole they could be considered markedly hypothyroid.

Uptake of a tracer dose of I^{131} in hypothyroid and control fish

The most promising means of evaluating the tracer data appeared to be a simple comparison of rate of activity loss in the thyroid region of the treated (hypothyroid) fish with that of the controls. In the first of two groups run to test the screening method, the initial readings were made four hours following the injection of I^{131}. In the hypothyroid fish, the counting rate ranged from 193 to 398 counts per minute, with a mean of 297 counts per minute. The controls ranged from 231 to 465 counts per minute, with a mean of 345. Since there was already a drop in the activity in the hypothyroid fish, as compared with the controls, a second group of fish was screened in the same manner, but taking the initial reading at one and a half hours, rather than four. Here the range in the hypothyroid fish was 313 to

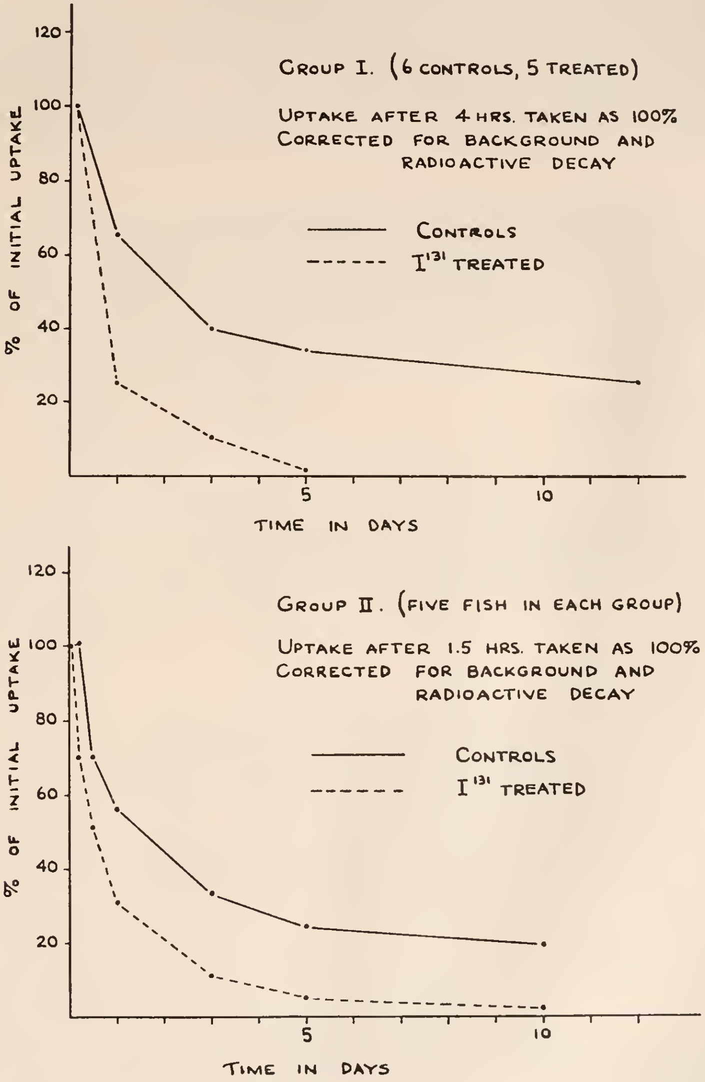

FIGURE 2. Activity loss in control and treated fish following a tracer dose of 2.5 μC of I^{131}.

447 counts per minute, with a mean of 383. The controls ranged from 315 to 507 counts per minute with a mean of 385. However, in both groups the subsequent readings on each fish were converted to percentage of its initial reading, the best way to take into account the variations between individual fish resulting from weight differences, thyroid activity, or unavoidable variations in dose.

While the activity of hypothyroid fish dropped immediately, the activity of the controls continued to increase for several hours before beginning to drop off. The peak appears to be around three to four hours following injection. The two sets of curves show essentially the same pattern: the hypothyroid fish losing activity quite rapidly while the controls lose it at a much slower rate (see Fig. 2). The mean values of the retained activity in the treated and control fish showed the greatest difference three days following the injection of the tracer dose; thus in the following screening of treated fish, the activity on the third day, as per cent of initial uptake, was the basis on which evaluation of thyroid destruction was made.

TABLE I

Blood chloride of hypothyroid and control fish kept in sea water and fresh water for 17 days

Group	No.	Range, gm.	Mean	s
Sea water hypothyroid	7	.730–.862	.792	.045
Sea water controls	6	.731–.875	.804	.055
Fresh water hypothyroid	7	.432–.743	.655	.113
Fresh water controls	6	.579–.751	.683	.068

Those treated fish whose activity approached or exceeded the mean activity of the controls were not used in later physiological experiments. However, all fish were subjected to a histological examination of the thyroid region following autopsy, and a comparison of the histological picture with the tracer readings was made.

Survival in fresh water

The experiment on osmoregulation was to have continued for six weeks following the abrupt transfer of the experimental fish from sea water to fresh water. However, after two weeks, three of the sea water hypothyroid fish had died, and three of the fresh water hypothyroid fish appeared very ill, showing such symptoms as tremors and difficulty in breathing. While these reactions occurred only among the treated fish and appeared to be a result of hypothyroidism, the occurrence of a sick fish among the sea water controls raised the possibility of some sort of infection, and the experiment was ended before any more fish succumbed, seventeen days after the transfer to fresh water.

Although the difference in salinity, *per se*, did not appear to influence survival, there was a definite correlation between the degree of thyroidectomy (based on histological evaluation) and survival in either fresh or salt water. Seven fish which died either before or during the experiment, had either no visible follicles, or extremely few.

Blood chloride

All fish in fresh water showed a definite drop in blood chloride (about 24 per cent), but within each group (*i.e.,* salt and fresh water) there was no significant difference between the hypothyroid and control fish (see Table I).

TABLE II

Growth of treated and control fish during nine months following the beginning of I^{131} treatment

Group	Length incr. %	s	Weight incr. %	s
Hypothyroid	11.71	3.9	54.66	23.1
Controls	13.02	7.0	57.74	29.9

Growth

Since there was no increase in length, but a significant weight loss during the experiment on osmoregulation, the readings used for growth measurements were those taken at the beginning of the I^{131} injections (October 13, 1956) and at the beginning of the osmoregulation experiment (July 3, 1957). The results show no significant difference in growth rates, either in length or weight, between the hypothyroid fish and the controls (see Table II).

TABLE III

Gonadosomatic index of hypothyroid and control fish kept in sea water and fresh water for 17 days

Group	GSI	s	GSI (comb.)	s
Sea water hypothyroid	3.09	1.57	2.47	1.62
Fresh water hypothyroid	2.14	1.39		
Sea water controls	2.51	1.49	2.91	1.20
Fresh water controls	3.01	.69		

Effect on other organs or tissues that may be dependent on thyroid function

The gonadosomatic index (GSI = testis weight/body weight × 100), calculated for each group separately, as well as for the combined hypothyroid and combined controls, showed no significant differences between any of the groups (see Table III).

Differences in the blood picture were to be found in the combined groups of sea water and fresh-water fish, rather than between hypothyroid and controls. The greatest difference appeared in the hemoglobin, with the sea water fish showing somewhat higher values. There was also a slightly higher red cell count in the sea water fish. The white cells showed a very puzzling drop in the sea water hypothyroid group, which cannot be accounted for. The thrombocytes show no significant differences (see Table IV).

Table IV

Hemoglobin titers, red cell, white cell and thrombocyte counts of hypothyroid and control fish kept in sea water and fresh water for 17 days

Group	Hb%	s	RBC*	s	WBC	s	Thromb.	s
S.W. hypothyroid	60	11.3	4.32	.69	5.54	.48	12.7	1.9
F.W. hypothyroid	55	11.4	4.11	.54	9.92	3.5	12.6	1.8
S.W. controls	63	7.9	4.64	.83	9.21	3.5	14.0	4.0
F.W. controls	50	13.6	4.51	.86	9.20	1.5	14.2	2.4

* RBC in millions, WBC in 1000's, Thrombocytes in 10,000's.

Discussion

Osmoregulation

There is good evidence to support the idea that the thyroid gland plays some role in the salinity tolerance of fish. It is well known that many species of fish show an activated thyroid gland during spawning migration from the sea to fresh water; experimental work of Olivereau (1948) and Leloup (1948) on several species of marine teleosts has shown that there is a strong activation of the thyroid gland with decreasing salinity of the medium; Fontaine and Callamand (quoted by Fontaine, 1956) have shown that thyroxine injections increase the survival time of several marine fish when transferred to fresh water. Thus it was of interest to test the salinity tolerance of hypothyroid fish, in terms of survival time and blood chloride concentrations.

Survival time, however, proved to be a function of degree of thyroidectomy, rather than of the salinity of the medium. Almost all of the treated fish which died during the course of the experiment were those with no follicles, or extremely few.

The blood chloride concentrations were obviously a function of the salt concentration of the medium. All fish in fresh water showed a definite drop in blood chloride (about 24 per cent) but there was no significant difference between the hypothyroid fish and the control fish. Burden (1956), on the other hand, found no change in blood chloride concentration of *Fundulus* kept in fresh water for eight days. Since the same method was used for the chloride determinations reported here, Burden's higher results might be attributed to the difficulty in determining the end point when using non-deproteinized serum. Sex and season were the same, and variations attributable to such causes are excluded. However, Burden's experiments were made at a lower temperature (15° C.) and this may have contributed to a slower period of adjustment to the new external environment. It is possible that there is a gradual chloride loss which was not detectable during the short period of time employed by Burden, although V. S. Black (1948) demonstrated a loss of body chloride in *Fundulus heteroclitus* transferred directly from sea water to fresh water, with a stable level of about 60 per cent reached after the fourth day. Bergeron (1956) has shown that *Fundulus* maintains a constant blood osmotic pressure in both salt and fresh water, confirming earlier work of Garrard (1935). It may be that the osmotic pressure is maintained by an exchange of carbonate ions for chloride, since the alkali reserve of the blood of marine fish is lower than that of fresh water species, and there is a relative decrease in the bicarbonate ion when migrating eels are transferred to sea water (Drilhon and Florence, 1936; Fontaine and Boucher-Firley, 1934). Work of Koch and Heuts (1942) and Heuts (1943) showed that changes in serum osmotic pressure of mature sticklebacks transferred to sea water could not be entirely accounted for by changes in blood chloride. In this light, further experiments seem to be called for to determine the rate of blood chloride loss following transfer from sea water to fresh water, and the factors involved in maintaining osmotic pressure, with special emphasis on the alkali reserve.

Other effects

The hemoglobin and red cell counts, like the blood chlorides, indicate a dependence on the medium, with no apparent influence by the presence or absence of thyroid tissue. While the increased values found in the sea water fish may be accounted for by the lower oxygen content of that medium (see Prosser, Barr, Pinc and Lauer, 1957), or by an increased energy demand for fish in higher salinities, as found by Hickman (1958) in *Platichthys stellatus,* the starry flounder, the differences are small and cannot be considered significant.

Other than the effect on survival, where the actual cause of death is unknown, there was no apparent effect of hypothyroidism on any of the physiological processes studied here. Growth rates were not affected by the hypothyroid condition, nor was there any effect on gonadosomatic index.

The literature devoted to thyroid regulation of growth in teleost fishes is confusing. With the species *Lebistes reticulatus* alone, anti-thyroid drugs have been reported to retard growth (Hopper, 1950, 1952; Gaiser, 1952; Vivien and Gaiser, 1952; Smith, Sladek and Kellner, 1953), while Fortune (1955) found no effect on growth in either *Phoxinus* or *Lebistes* (see Pickford and Atz, 1957). Possibly collateral toxic effects of the anti-thyroid drugs may be responsible for the retardation of growth, and it is of interest that in salmon parr thyroidectomized with I^{131} no effect on growth was found (La Roche and Leblond, 1954). Thyrotropin injected into hypophysectomized *Fundulus* was found by Pickford (1954) to have no effect in restoring growth, indicating that the thyroid at least has no direct effect on growth. However, in such fish, as in hypophysectomized rats, thyroid stimulation undoubtedly enhances the response to exogenous growth hormone (Pickford and Atz, 1957, p. 99).

Data concerning the role of the thyroid in sexual maturation are no less conflicting than those on growth. However, studies with anti-thyroid drugs strongly indicate that the thyroid is instrumental in the maturation of the gonads (reviewed by Pickford and Atz, 1957). While Barrington (1954) and Fortune (1955) found that *Phoxinus* could reach sexual maturity despite treatment with thiouracil, it is possible that there was not complete inhibition of thyroid function, as in the work reported here, and that this minimal amount of hormone was sufficient to permit sexual maturation.

Screening method

There appears to be a considerable discrepancy between the evaluation obtained with the tracer technique and that from the histological examination, based on apparent number and size of follicles and cell height. Since there was a time lapse of approximately three months between the time of tracer screening and autopsy, the most plausible explanation for the difference is the regeneration of thyroid tissue in that period. Olivereau (1957), in her work on radiothyroidectomy of eels, found that in fish of about 40 grams that had received a total of 1000 μC of I^{131} in three doses, functional thyroid tissue had already regenerated after two months, and seven months later she found complete absence of the thyroid in only three out of fifteen fish. It would thus be advisable to repeat the tracer screening just prior to autopsy.

SUMMARY

Thyroidectomy of *Fundulus heteroclitus* was attempted with the use of radioactive iodine, administered in three doses of 25, 15, and 10 μC per five-gram fish at intervals of five weeks. A screening method was developed whereby the degree of thyroidectomy could be determined by the rate of activity loss in the throat region of the fish following a tracer dose of I^{131}. Thyroidectomy was not complete, and in some cases there was considerable regeneration. However, in general, the resulting condition was one of extreme hypothyroidism, and physiological studies conducted with these fish yielded the following results:

1. There was no special effect on the fishes' ability to survive in fresh water, although there seemed to be an impairment of their ability to survive at all, in either salt or fresh water. Deaths occurring during the experiment in general involved fish with very few follicles or no thyroid tissue remaining.
2. Blood chloride titers were a function of the salinity of the medium and were not affected by lack of thyroid.
3. Hemoglobin titers and red cell counts indicated an effect of the medium, and were not influenced by lack of thyroid.
4. Hypothyroidism had no effect on growth, either in length or weight.
5. Hypothyroidism had no effect on the gonadosomatic index.

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PROTOPLASMIC MOVEMENT IN THE FORAMINIFERAN, ALLOGROMIA LATICOLLARIS; AND A THEORY OF ITS MECHANISM

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Protoplasmic streaming in reticulopodia has been described by numerous investigators, most of whom have commented on what an impressive phenomenon it is. One of the most detailed but condensed descriptions is that of Leidy (1879) who stated (pp. 279–280):

"In the emission of the pseudopodal filaments of *Gromia terricola,* the protoplasm pours from the mouth of the shell in a slow manner, and gradually envelopes the body. . . .From the protoplasmic envelope delicate streams extend outwardly, at first emanating from the front; they more or less rapidly multiply and radiate in all directions. Gradually extending, they fork into branches of the utmost tenuity. Contiguous branches freely join or anastomose with one another, and thus establish an intricate net, which in its full extent covers an area upward of four times the diameter of that of the body of *Gromia.* The pseudopodal net incessantly changes, putting forth new branches in any position, while others are withdrawn, diminishing and disappearing in one spot, while it spreads and becomes more complex in another spot.

"*Gromia terricola,* with its pseudopodal net fully spread, like its near relatives, reminds one of a spider occupying the center of a circular web. If we imagine every thread of the latter to be a living extension of the animal under the same control as its limbs, the spider would be a nearer likeness to the *Gromia.* Over each and every thread of the pseudopodal net, *Gromia* has a complete control as if the threads were permanently differentiated limbs acted on by particular muscles, and directed in their movements by nervous agency. Threads dissolve their connection and are withdrawn; new ones are formed and establish other connections; they bend; they contract into a spiral; they occasionally move like the lashing of a whip, and indeed produce almost every conceivable variety of motion. Not infrequently spindle-like accumulations of protoplasm occur in the course of the pseudopodal threads. Sometimes, through the conjunction and spreading of several of the latter together, islet-like expansions occur; and become centres of secondary nets.

"The pseudopodal extensions of *Gromia* consist of pale granular protoplasm with coarser and more defined granules. The latter are observed to be in incessant motion along the course of the threads, flowing in opposite directions in all except those of greatest delicacy."

Another excellent description of foraminiferan movement was provided by Jepps (1942) for *Polystomella.* She described more or less the same activity described by Leidy for *Gromia.* In regard to the pseudopodia she states (p. 624) that they "wave about like minute feelers, bending, undulating, quivering, and putting out side branches which meet and fuse and so establish the reticulum". . . and that (p. 625) "The pseudopodia show a fairly high degree of stiffness; they extend in a straight line as a rule, and may stretch unsupported through the water for a distance at least two to three times as great as the shell diameter." (The greatest shell diameter she mentioned was about 1.8 mm.)

All of the above statements of Leidy and Jepps concerning streaming in *Gromia* and *Polystomella* are equally true of streaming in *Allogromia laticollaris,* and presumably apply to most, if not all, organisms with reticulopodial nets, with the exception that some species have been described as being much more active than others, but with no real differences described in the general type of protoplasmic activity, sometimes described as "filament streaming" (Fädchenströmung, Engelmann, 1879).

It has been recognized that the theory of flow caused by differential pressure in the plasmasol, which is so well accepted for *Amoeba proteus* and related genera and for Mycetozoa (reviews, Seifriz, 1942; De Bruyn, 1947; Bovee, 1952; Noland, 1957; also Kamaiya and Kurodo, 1958), would not explain the streaming of Foraminifera. This was pointed out clearly by Sandon (1934) who recognized the significance of the fact that there is no tube of plasmagel and no evidence of sol-gel reversibility in the active pseudopodia, and stated that it was time for another explanation to be developed. Considerable doubt concerning applicability of the pressure differential theory was also expressed by Noland (1957). So far no detailed alternative theory has been proposed to explain the streaming in Foraminifera, although the direction that such a theory should take was clearly pointed out by Noland (see below).

The purpose of the present paper is 1) to extend the observations of Leidy, Jepps, and others on movement in reticulopodial nets, 2) to postulate an active shearing mechanism for this type of movement, based on observations by the present authors and by previous investigators and on recently discovered facts concerning the mechanism of muscular contraction, and 3) to discuss briefly the taxonomic implications of the existence of two basic types of protoplasmic movement in the Sarcodina.

These two basic types of protoplasmic movement are: a) flow of plasmasol caused by differential pressure, which in turn is caused by contraction of a plasmagel cortex, and b) flow presumed to be caused by the newly postulated active shearing force between two adjacent oppositely moving gel-like filaments of protoplasm in the same pseudopod and in the absence of a typical plasmagel cortex.

Allogromia laticollaris was described by Arnold (1948) who has studied its movement and dispersal (Arnold, 1953), variation and isomorphism (Arnold, 1954), and life history and cytology (Arnold, 1955). Arnold's published studies on movement have included changes in location of the organism as a function of time and in relation to other organisms and in relation to environmental influences, but do not include a study of movement in the sense of protoplasmic flow and the mechanism of flow, as used in the present paper.

MATERIAL AND METHODS

Allogromia laticollaris, originally described from Florida (Arnold, 1948), is a common foraminiferan of the sea coasts of the United States. It is a large organism, possessing a globular test with an average diameter of 200 to 400 microns, and a reticulopodial net of several times this diameter. It has been maintained continually in laboratory culture by Dr. Zach Arnold, who has kindly provided us with the ancestors of the organisms used in this work. The only requirements for prolific culture are sea water, nitrate and phosphate, some soil humus, a light source to permit growth of algae used as food, and containers, such as finger bowls. *Allogromia* is tolerant of heat and will reproduce within the range of 15 to 34° C.

Observations were made with the aid of ordinary bright field microscopes, a Zeiss inverted microscope with phase and bright field equipment, and a Leitz variable phase microscope, which also permitted dark field observations.

Micrurgical experiments were performed on organisms on open slides in a drop of sea water surrounded by a vaseline ring. Coverslips were added later for critical microscopic observations.

Results

1. *General protoplasmic arrangement*

The protoplasm of *Allogromia,* like that of all Foraminifera, lies 1) within the test, 2) around the outside of the test so that the test is more or less internal, and 3) in a network of pseudopodia, usually called reticulopodia, which may and usually do fuse peripherally to form complicated anastomoses, with numerous nodes, of various and continually varying sizes, all of which results in the formation of a reticulum, sometimes of very great complexity.

The following discussion applies specifically to the reticulopodia of medium or small diameter, *i.e.,* under 5 μ. Near the body of the organism some of the pseudopodia are larger, but at a short distance from the body those over 10 μ are rare. It appears as if some of the larger pseudopodia are fundamentally bundles of the smaller ones. Some of the following statements, *e.g.,* those concerning absence of a non-moving central core, and possible absence of a cell membrane, do not necessarily apply to the pseudopodia of larger diameter and definitely do not apply to the main body of protoplasm or even to the larger nodes of the reticulum.

Attachment to the substratum occurs in some of the more peripheral nodes of the reticulum, and presumably in some of the small peripheral masses of protoplasm sometimes found near the ends of the pseudopodia and which do not have side branches of reticulopodia, and sometimes under the main body of the organism. The active portions of pseudopodia under 5 μ are not attached directly to the substratum for much, if any, of their length, and many of them certainly are not attached to the substratum at all, except indirectly through the nodes or main body of the animal.

Branching and rebranching may occur throughout the length of the reticulopodia, *i.e.,* for several millimeters or more. However, a very high degree of branching occurs at the region where the mass of protoplasm merges from the opening in the test, so that the pseudopodia are seldom more than 10 μ in diameter at the base as they emerge from the general protoplasmic mass. In the early stages of emergence or the later stages of withdrawal the appearance of the numerous pseudopods sometimes resembles a tuft of brush bristles being pushed free end foremost out from the body or being drawn into the body. A similar description is given by Doflein (1916) for *Gromia.*

2. *Protoplasmic or filament streaming*

Allogromia is able to extend a network of radial granular reticulopodia from its test as far as 15 millimeters in a circular pattern into its environment. In addition to the radial pseudopodia there are pseudopodia which form cross-connections between one radial pseudopodium and another. Individual pseudopodia have an average diameter of 2–5 μ, but some have a diameter of considerably less than 1 μ. Structure of a small branched pseudopodium is shown in Figure 1.

Streaming can be determined by observing the movement of granules. Previous investigators of the Foraminifera have pointed out that streaming is usually in two directions simultaneously in the same pseudopodium. One important point is that in our observations we have found that streaming is *always in two directions simultaneously* in every pseudopod as shown in Figure 2. In radial pseudopods one stream goes *toward* the body and the other *away* from the body, and in pseudopods that form cross-connections in the reticulum, each stream goes in the direction opposite from the other. We have never been able to observe streaming in one direction only. In certain of the smaller reticulopods it sometimes may appear superficially that movement is unidirectional because one stream may be out of focus or has fewer granules. However, upon careful focussing with bright field objectives and more easily with phase objectives we have *always* been able to find movement in the opposite direction even in the finest pseudopods. This is an observation that has great theoretical importance as far as the proposed mechanism of filament streaming is concerned.

In the medium sized and smaller pseudopodia of *Allogromia,* the protoplasmic material consists of two parts, each more or less the shape of a semi-cylinder, but also possibly flattened. In radial pseudopodia one semi-cylindrical portion is streaming in the outward or distal direction and the other semi-cylindrical portion is streaming in the inward or basal direction. We have not been able to detect a gel tube in any of the pseudopodia, even in those of large diameters. Neither is it possible to see the line of demarcation between the two oppositely moving layers.

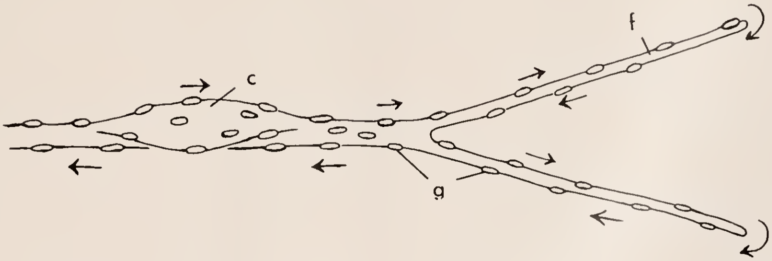

FIGURE 1. The general shape and structure of the distal portion of one of the finer pseudopodia, with a single bifurcation into branches about one micron in diameter. Arrows show movement of the granules (g), and of a small cytoplasmic mass (c), all of which are attached to the actively moving filament (f).

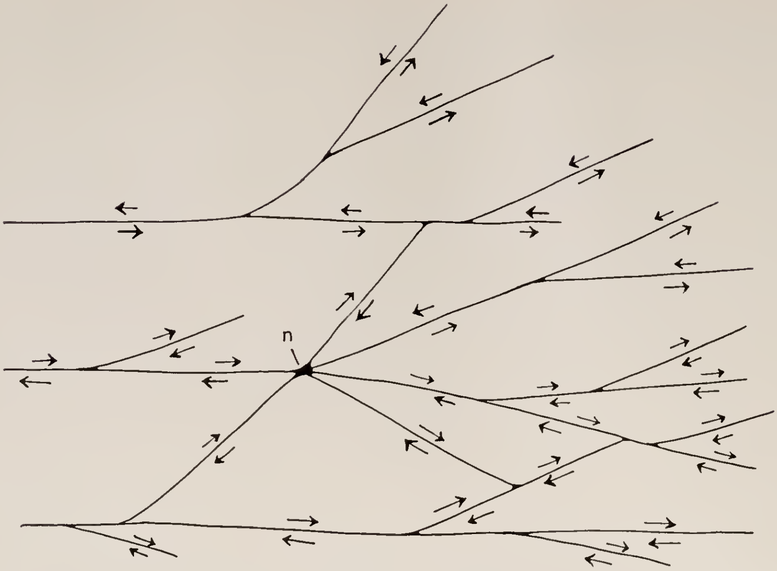

FIGURE 2. Anastomoses of three reticulopodia. Body of organism at left. Arrows show direction of streaming. Node (n) mentioned in text.

Reticulopodia are extended by a greater flow in the outward direction and at least usually are retracted by a greater flow in the inward direction. However, by simple visual observation it is not possible to say definitely whether greater flow is obtained by greater velocity or by greater cross-sectional area in one of the two directions.

The reticulopods are capable of great activity. They can bend and twist or even move laterally as they are extended; they can split, forming Y junctions, with base toward the body, and can anastomose, forming inverted Y junctions, with the base toward the periphery. One pseudopodium may split to form two pseudopodia, which may be parallel to or divergent from each other, with active two-way streaming in both. Also, new side projections or branches may be pushed out from a pseudopodium, and these projections are sometimes *carried* by the protoplasmic stream, and always exhibit two-way internal streaming themselves. The simple Y junctions can migrate along the pseudopodium in either direction and combine with other junctions to form X's and more complex types of junctions. Activity, always meaning double streaming, regardless of whatever else might be included, is at least almost continuous, and no pseudopodia appear to be in a condition of rest; those that are not streaming are invariably moribund. This constant state of activity, with continual splitting and anastomosing of pseudopodia, with bending, twisting, and lateral movement, can readily give the teleological impression, mentioned by Leidy (1879) that protoplasmic flow is under a most delicate if not deliberate central control, of the organism.

It is assumed that the streaming protoplasm of the reticulopodia is in a gel rather than a sol state. This assumption arises from the following observations:

1) Pseudopodial extensions only a few microns in diameter may be extended at almost any angle into the medium, for at least hundreds of microns, in a relatively straight line. They are more numerous along or near the substratum, but they are by no means limited to the substratum, and they certainly are not necessarily attached to the substratum.

2) The pseudopodia are not readily bent or shoved aside when bumped by ciliates, microcrustacea, or other swimming organisms, and exhibit a certain degree of rigidity.

3) The granules in the pseudopodia of small diameter, at least under simple visual observation (and with only occasional exceptions, mentioned below), seem to move in the stream without changing their relative positions and at least about the same distance apart.

The terms "gel" and "sol" are relative ones used to describe different ranges of viscosity. Consequently, the line of demarcation between them is not a sharp one. As used here, the word "gel" denotes a viscosity high enough to permit retention of form under a considerable degree of stress, for example, under the conditions mentioned above. In order to explain the observed phenomena the assumption of a considerable degree of rigidity seems necessary, and this degree of rigidity seems far in excess of that of the sol and comparable to that of the gel of *Amoeba proteus*. We have planned a cinephotomicrographic analysis in order to elucidate this point. The simple criterion of making an estimate of the degree of Brownian movement is not applicable because of the continuous streaming. The usual methods of measuring viscosity cannot be used for the same reason.

Another important observation is that at least in all pseudopodia under 5 μ in diameter *all* of the visible granules are streaming. *There is no tube of gel.*

Neither is there any appreciable space, or any other evidence whatever, for a hyaline layer outside of the moving stream. The stream consists of a hyaline gel material to which the large granules are attached.

Furthermore, there is no evidence for a central core of refractive non-moving protoplasm (stereoplasm), even when the pseudopodia are observed with the aid of dark field and phase equipment. This observation is in agreement with those of Doflein (1916) on *Gromia dujardini* in which he was unable to find a central core, and are in contrast to the observations of Doflein (1916), Schmidt (1937), Jepps (1942) and others who have described central cores in other genera of Foraminifera, which are not so closely related to *Allogromia*.

The granules of the reticulopodia are 2 to 4 μ in diameter, which may be greater than the average diameter of the pseudopodium in which they are contained. Therefore, they seem to be attached to rather than contained within the clear streaming material which comprises the actively moving portion of the pseudopodia. Sometimes the granules traveling in one direction can be observed to bump into granules traveling in the opposite direction, and to hit with such force that they become detached and then attached to the oppositely directed thread, thereby reversing their direction of movement. Less vigorous bumping may result in a backward shift of the position of a granule in relation to others in the same stream, but without a shift into the opposite stream.

Frequently there are small masses of protoplasm, more or less spindle shaped, up to several times the diameter of a pseudopodium, which migrate along with the protoplasmic stream, either outward eventually to fuse with one of the nodes and build up a secondary protoplasmic center from which more pseudopodia may radiate, or inward toward the body. This has been described by Leidy (quotation above) and others for other species, and is shown in text Figure 1 (C). Such spindles are few in newly formed reticula but may be numerous in older ones.

Cross-connections may remain more or less in one position or may move laterally, that is, basally or distally, depending upon whether both ends are involved with outgoing or with ingoing streams, or may be pulled diagonally, with one end moving basally and the other end distally, if connected to one outgoing and one ingoing stream. Lateral movement of cross-connections, either basally or distally, but usually basally, is very useful in the engulfment of food particles.

Streaming granules can be seen going through the nodes of the reticulum in definite pathways, so that a node with a dozen or more or even with only a few radiating pseudopodia (as in Figure 2, n) seems a jumble of moving granules. These bump into each other continually, and therefore may seem superficially to be merely undergoing Brownian movement. However, closer examination under high magnification reveals that the pathways are quite definite and that most of the granules are moving in single file. Furthermore, under dark field illumination it seems at times as if these pathways are traced by very fine clear fibers to which the granules are attached, exactly as in the very fine pseudopodia described above.

Likewise, near the base of the larger pseudopodia which have many branches, the streaming is in the form of many narrow pathways, lying side by side, some with granules moving single file and some obviously in multiple lines, some directed basally and some distally, but usually with the lines well mixed in arrangement and not completely segregated according to direction. Similarly the pseudopodia intermediate in diameter seem to be made up of the same paired filaments of each of its branches, so that it is entirely probable that all except the finest pseudopodia are fascicles of the finer units, often with a partial fusion of filaments moving in the same direction, but certainly often without a complete fusion. This lack of complete fusion can account for the existence of more than one speed of streaming, as sometimes seen in the pseudopodia of intermediate and of larger diameter.

For these reasons it seems as if the protoplasmic threads to which the single rows of granules are attached are continuous, both in at least some of the nodes of the reticulum and in the larger pseudopodia. Therefore, in a certain sense and to a very considerable degree the paired hyaline filaments of the finer pseudopodia may be considered the fundamental structural units of the reticulum.

These fundamental streaming units, considerably less than a micron in diameter, are optically homogeneous as viewed with bright field, phase, and dark field objectives. Except for the bumps on the pseudopodia caused by the presence of granules the pseudopodia seem to be of quite uniform diameter throughout their unbranched portions, but they can differ in diameter from each other and are different in diameter before and after branching. We assume that this means that two or more of the paired fundamental units are fused to form all but the finest of the pseudopodia. In the smallest filaments there are fewer granules, but these granules can be traced individually as they move completely to the tip of the smallest clear filament and then turn 180° around the tip and start back toward the base of the filament.

Streaming is about 8 to 15 μ per second under conditions of our observations, but the results of modifying these conditions have not been studied.

The above description of filament streaming applies not only to the large adult organisms, but also to the smaller specimens, and even to the smallest ameboid forms that we have identified in cultures. Presumably this includes most of the stages of the life cycle.

Occasionally the end of a reticulopod may be turned back upon itself by extraneous forces and then begin to roll up into a spiral so the general form of the pseudopodium resembles a more or less flattened coil, with each turn in close contact and presumably fused with the adjacent turns, and with two-way streaming continuing for a number of minutes. The coiling seems to result when the tip of a pseudopod is bent so that it comes in contact with the outgoing protoplasmic stream. Two-way streaming continues in all parts of the spiral, and the coil continues to increase in size as long as contact is maintained only with the outgoing stream. It is possible that this is what Leidy meant when he stated (quoted above) "they contract into a spiral." Coiling requires about a minute, and a few minutes later the coil degenerates into a simple protoplasmic mass and then develops new pseudopodia.

3. *Flow on the reticulopodial surface*

Streaming of foreign material can easily be demonstrated on the surface of reticulopodia in *Allogromia* by use of a dye, *e.g.*, Evans brilliant vital red, which is insoluble in sea water. The dye particles, which may be ten or more times the diameter of the pseudopodia, stick to the protoplasmic surface and *flow along with the protoplasm* (Fig. 3). Individual dye particles may stick to either the distally or the basally directed streams and therefore may pass each other going in opposite directions. The same phenomenon can be demonstrated less colorfully by means of particles of finely ground glass. This is apparently a non-specific reaction, and occurs normally with all materials (primarily algae) that serve as the food source for the organisms. Normally food particles are carried by the basally directed stream until engulfed by the main body of protoplasm or by the distally directed stream until engulfed by one of the major distal masses in the network or until it is redirected into a basally directed stream.

4. *Movement of the entire organism*

When the organism is moving there is no apparent contraction of the anterior pseudopodia, as is well known for other shelled rhizopods (*e.g.*, *Arcella*). The anterior pseudopodia, which apparently pull the body and test forward, continue to have a rapid, and perhaps have a more rapid, two-directional streaming while the body is moving. The most reasonable explanation seems to be that the distal portion of the reticulum is attached to the substrate, that the distally streaming protoplasm is actually pulling the body forward, and that the motive power is the same active shearing process responsible for the streaming. The possibility should not be overlooked that movement may also involve some type of rapid contraction of the larger pseudopodia, as mentioned by Doflein (1916), Schmidt (1937), Jepps

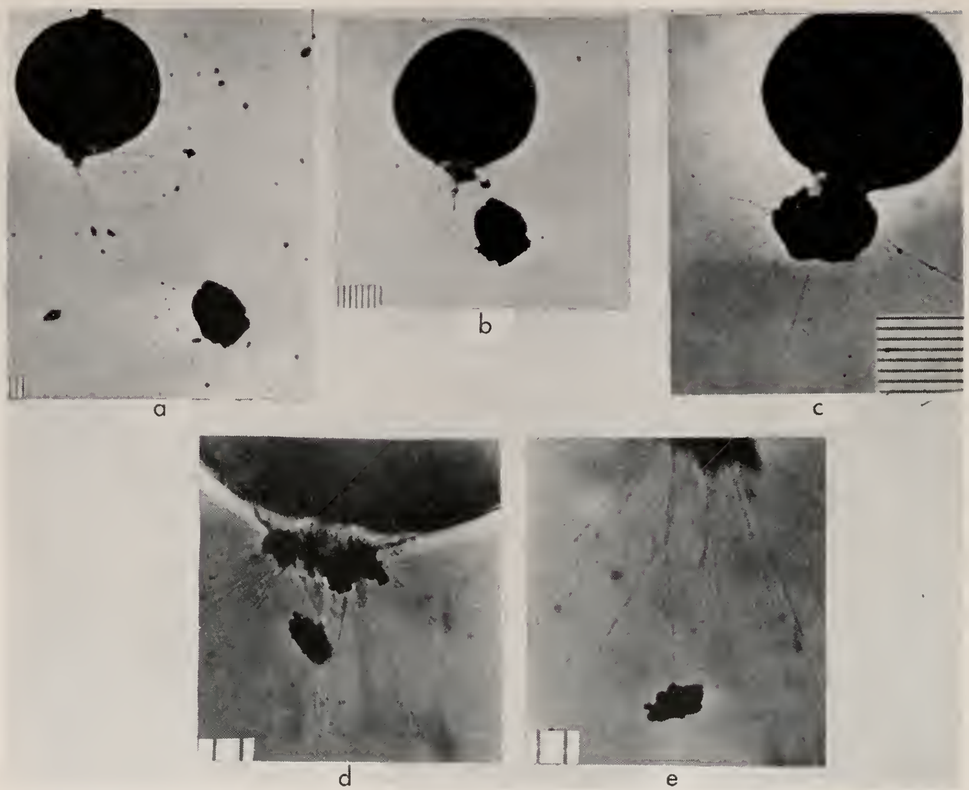

FIGURE 3. Photographs showing movement of dye particles attached to reticulopodia. *a*, Note large dye particles at lower right, and small ones scattered; *Allogromia* at upper left. *b*, Thirty minutes after *a*. Note movement of large particle toward opening in test of *Allogromia*. *c*, Thirty seconds after *b*. *d* and *e*, Dye particles moving along the pseudopodia. Note lack of engulfment of particle.

(1942), and others for other Foraminifera. However, we have not seen any movement which could be interpreted in this manner.

The tensile strength of active pseudopodia can be demonstrated by means of displacement with microneedles or movement of the medium. If a microneedle is entangled in some of the pseudopodia, the whole organism can be broken loose from the slide, leaving behind some fragments of protoplasm at the points of attachment, mostly in the peripheral portions of the net. Then the organism can be held by the microneedle attached to the pseudopodia while the slide is moved by the mechanical stage of the microscope. The pseudopodia which are not attached to the needle are dragged by the medium and trail as loose lines. When the direction of the slide is reversed the relative positions of the body and of the trailing pseudopodia are also reversed.

However, under these conditions the protoplasm in the pseudopodia attached to the needle, even if only a single pseudopodium, continues to flow, and in both directions.

5. *Formation and behavior of protoplasmic fragments or satellites*

We have confirmed the experiments of Grell (1956) that if the peripheral portion of a pseudopod of *Allogromia* is amputated, the fragment can fuse with the pseudopodial stump and again become part of the organism.

Furthermore, by repeated cutting of pseudopodia and removal of the main body of the organism we have been able to obtain small fragments of protoplasm. In small segments of a pseudopod, about 40 μ long, cut at both ends, two-way streaming was observed immediately after the cuts were made. This proves that the connection to the main body of the organism is not necessary for two-way streaming. However, such fragments soon become rounded, forming what we have termed "protoplasmic satellites." These satellites can persist for about forty minutes under conditions of our experiments. During this time they become stellate in appearance by extending several fine pseudopodia which are capable of bending, twisting, and anastomosing, and which exhibit two-way streaming. In small satellites most of the larger granules often remain in the central mass of the satellite and usually are rare in the pseudopodia (Fig. 4). The larger satellites have granular pseudopods. Satellites are capable of fusing with the parent organism and also with each other. Furthermore, upon disintegration the pseudopods of satellites have been seen to split into two filaments, free of granules.

Satellites also may be formed by rapidly crushing the organism between slide and coverslip. A rapid crushing causes most or all of the protoplasm of the body to dissolve in the sea water, but this does not necessarily result in solution of the uncrushed portion of the network. The larger nodes become the center of stellate protoplasmic masses, sometimes with dozens of radiating pseudopodia and numerous cross-connections, all of which exhibit two-way streaming. These may continue to stream for at least several hours. The nodes and the main body of these satellites also exhibit the definite granule pathways described for the intact organism. When the organism is crushed some of the pseudopodia may become completely isolated, and these also exhibit two-way streaming and may form small satellites similar to those obtained by micrurgical methods.

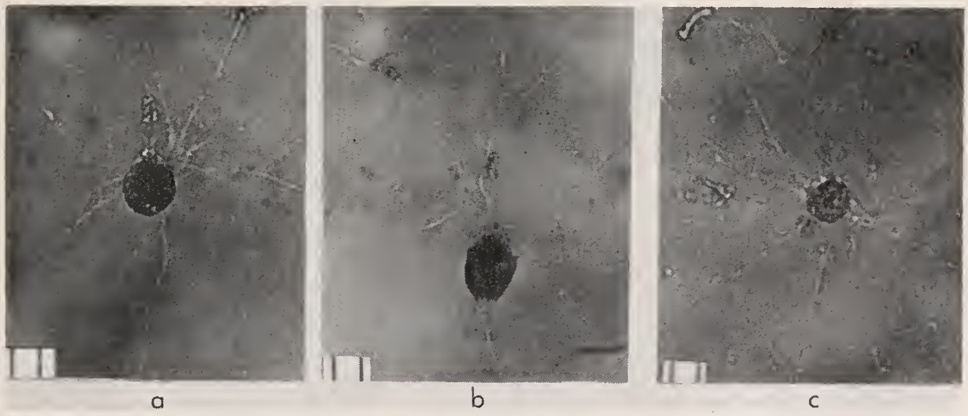

FIGURE 4, a, b, AND c. Protoplasmic satellites of *Allogromia*. Note anastomoses and orientation of pseudopods exactly as in intact organism.

Satellites which behave exactly as those described above can also be formed by quickly detaching the organism after it has extended pseudopods and attached them to the substrate, so that the attached ends remain attached to the substrate and become satellites.

In general, in so far as the size of the satellite permits, streaming in satellites is exactly the same as that in the intact organism.

Discussion

1. *Inadequacy of the pressure flow theory applied to Allogromia*

The idea that protoplasmic flow in *Allogromia* could be caused by differential pressure in a sol resulting from contraction of a partially surrounding gel tube does not seem to be in accord with the following facts:

1) Absence of a gel tube in the pseudopodia, and even the possible absence of a pseudopodial membrane (see below).

2) Presence of two-way streaming in all active pseudopodia, even those of smallest diameter, including all of the radial and all types of cross-connecting pseudopodia, both in the intact organism and in even the smallest protoplasmic satellites.

3) Presence of definite criss-crossing pathways for granules through nodes of the reticulum.

4) Presence of numerous parallel granule pathways in the larger pseudopodia, without segregation of pathways on the basis of direction, and without any evidence of gel tubes through which the granules could flow.

5) Presence of two-way streaming in freshly cut segments of pseudopodia.

6) Reversal of direction of granules at the tip of each pseudopodium.

Therefore, it is assumed that the theory of protoplasmic flow caused by differential pressure in a sol cannot apply to the protoplasmic flow of the reticulopodia of *Allogromia*. Likewise, it seems as if the pressure flow theory can not apply to the same general type of streaming in other Foraminifera, which has been described by other investigators. Therefore, a new theory is proposed.

2. *Theory of the mechanism of filament streaming in Allogromia*

The mechanism proposed to explain this type of movement is the existence of active shearing forces located in the reticulopodia between two paired filaments of protoplasmic gel, or rather between two portions of the same filament. These forces act longitudinally and oppositely and thereby produce the typical two-way streaming.

This is shown diagrammatically in Figure 5. In its simplest form the pseudopodium includes only two approximately semi-cylindrical, but possibly flattened, filaments of protoplasmic gel, labelled f_1 and f_2. Filament f_1 is the outgoing portion, and f_2 is the ingoing portion, as denoted by the large arrows. These are continuous at the tip of the pseudopodium and are therefore parts of the same filament. There may be some reorganization of the outgoing protoplasm at the tip before it becomes ingoing, but we have not been able to determine the degree of reorganization by simple visual methods.

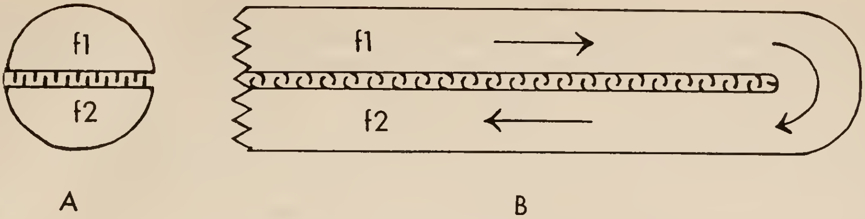

FIGURE 5. Arrangement of actively moving filaments (f_1 and f_2) of pseudopod. A, cross-section; B, side view. Direction of movement shown by arrows. The moving material is assumed in the diagram to be in the form of a semi-cylindrical filament, turned back upon itself at the tip, with the flat surfaces opposed. The shearing force is assumed to be between the adjacent surfaces and is designated by the short curved lines.

The small hook-like structures represent the active shearing mechanism, which at present is completely unknown. It must act in the direction indicated by the small arrows, and it must be capable of acting against the same mechanism on the opposite semi-cylinder. All other properties of the mechanism remain undetermined. As diagrammed here one could imagine the gel filament with its active mechanism to be similar to a millipede of relatively enormous and indeterminate length, folded back upon itself at the tip and with the legs of one portion of the body pushing against those of another portion throughout the length of the pseudopodium.

The basic idea is not new, but was proposed in somewhat different form by Noland (1957), in a general manner and without detailed application. In discussing the structure of protoplasm in *Amoeba proteus* Noland stated (p. 4), ". . . the endoplasmic molecules, some of them at least, must be quite linear in form. If one lets his imagination have free play he might compare the molecules of the plasmasol to a writhing mass of centipedes, each hanging on to his neighbors with a leg or two, but often losing hold and grasping any other one that comes with reach. Thus the whole mass, though moving, would maintain a certain coherence, so that a tug at one centipede would be communicated some distance into the mass." Furthermore, in expressing doubt that the pressure differential theory could be applied to reticulopodia, he stated (p. 6), "To revert to our centipede similes, what we need are molecules that can orient themselves in one direction and crawl forward on any solid surface, while others of the same sort crawl forward on the back of the first layer."

The present proposal, aside from substituting millipedes for centipedes, but otherwise continuing the simile, assumes that instead of one animal moving on the back of another, that the distally bound millipede is merely a more posterior segment of the basally bound millipede and that "they" are moving with the feet of one segment pushing backward against the feet of the other. One very important development since Noland wrote his paper on this subject is that it has now been definitely proven that in striated muscle we do have a mechanism that can "crawl" in the sense used by Noland, not on any solid surface, but on a highly specialized surface (review, A. F. Huxley, 1957). The existence of such a solid foundation changes what might have been considered a rather speculative hypothesis into a well founded theory, worthy of considerable development and investigation.

The completely unknown part of the mechanism is what the "millipede" uses for legs. In striated muscle the connections from the myosin fiber to the actin fiber, that is, the connections which actually exert the force involved in the sliding motion, may be seen with the aid of an electron microscope. It is also obvious from chemical and cytological studies that ATP is used by the sliding mechanism (review, A. F. Huxley, 1957). Otherwise the status of definite knowledge is not much more advanced for muscle than for reticulopodia; the actual mechanism of the "legs" is unknown in both. Furthermore, in reticulopodia we can observe this sliding of one fiber upon another continuously by means of an ordinary microscope for hours, or even for days, or as long as the patience of the observer persists.

In addition to the active shearing force there obviously must be some mechanism that holds the two active surfaces together and more or less in contact with each other. If the active force is transmitted through protein bridges similar to those which can be demonstrated in electron micrographs of striated muscle (H. E. Huxley and Hanson, 1955; A. F. Huxley, 1957), then the same bridges serve both purposes, and the two mechanisms are really two aspects of the same.

It is obvious that the word "streaming" when used in conjunction with the present theory of filament streaming is used in a very special sense, and only for historical reasons. It does not mean the streaming of a sol, as in *Amoeba* or in *Physarum,* or even in Foraminifera as assumed by previous investigators. It means the longitudinal movement of gel-like filaments, or threads, of protoplasm, carried along by active processes on the adjacent surfaces of the threads themselves, that is, on the surfaces between the two parallel threads which are "streaming" in opposite directions.

One unanswered question concerning which we have no evidence is whether the filaments of gel remain essentially as filaments when the material is all inside of the body, or whether they are in the form of undifferentiated protoplasm, which is re-formed into filaments when the pseudopodia are re-extended. The tuft-like appearance of many simultaneously emerging pseudopods might suggest that the structure is not completely destroyed within the body.

Another unanswered question is how the organism is able to extend or retract all pseudopodia simultaneously. Is there a central control of these movements? Perhaps, and perhaps not. However, if there is a central control, the present theory offers a very simple explanation. The outgoing thread of gel must be formed from, or at least released from, the body of the animal, and the ingoing thread must be incorporated into the body, either as a stored filament or as dedifferentiated protoplasm. Extension and retraction could be controlled very simply if there were control of either the release or the re-incorporation of the filaments, or of both of these processes.

One characteristic of reticulopodia which perhaps should be emphasized is that in at least all of the finer and possibly in all of the pseudopods there is no tube of plasmagel as in *Amoeba proteus* and in *Physarum.* This general characteristic has been noted by most of the earlier investigators (*e.g.,* Doflein, 1916; Schmidt, 1937; Jepps, 1942). The fact that a gel tube is absent is of great theoretical importance from the viewpoint of explaining the mechanism of movement. The absence of a gel tube definitely rules out the pressure differential theory, unless one assumes that the cell membrane is mechanically capable of performing the functions ordinarily assigned to the tube. The question of the structure of the membrane and even the possibility of its non-existence on the reticulopodial surface is discussed below.

Leidy stated that streaming was always in two directions in all pseudopodia "except those of greatest delicacy." It is possible that he was not able to detect oppositely directed movement because of a scarcity of granules in the opposite stream. However, it is also possible that some of the "pseudopodia of greatest delicacy" are formed by splitting of an undirectional thread from a pseudopodium and that this thread is merely pushed passively into the medium. The present authors have seen a few temporary pseudopods which could be interpreted in this manner. However, such a thread, at least according to the present theory, could not develop into an active pseudopodium without developing a return flow.

The theory, as outlined above, will explain all of the facts as we know them, including the six items listed above (Discussion, section 1) which cannot be explained by the theory of differential pressure in a sol.

3. *Application of the theory to other materials*

It seems as if the present theory could apply in slightly modified form to the reticulopodia or rhizopodia which have a central core of stereoplasm and also to axopodia, which have a well defined axial filament. From one point of view the only change necessary is to assume that the active mechanism is capable of moving along the surface of the stereoplasm or the axoneme rather than only along the active surface of an oppositely directed filament. This is the equivalent of introducing Noland's idea of molecules crawling upon a solid surface. Another possibility is that the active shearing occurs not between the rheoplasm and the stereoplasm or axoneme, but between the outgoing rheoplasm and ingoing rheoplasm where they come in contact with each other, peripherally to the stereoplasm or axoneme. The central cores and the axonemes may serve architectural functions, but since they do not exist in *Allogromia* it is obvious that they are not necessary to explain either the stiffness or the streaming that exists in the reticulopodia of *Allogromia*.

Previous investigators (*e.g.*, Doflein, 1916) have pointed out that as the pseudopod is extended, more stereoplasm is added at the distal end of the core and that this must come from the rheoplasm. If so, and if the present theory also applies to species which have a central core, the core is composed merely of temporarily inactivated fibers of rheoplasm, that is, of the hyaline material without the attached granules.

Many of the stamen hair cells of plants have two-way streaming in fine threads of protoplasm which go across (that is, through) the cell vacuole (*e.g., Zebrina, Tradescantia*). In many instances it is obvious that streaming is in both directions. However, streaming sometimes appears to be undirectional because the cytoplasm from one direction has few granules and is therefore difficult to detect by observation. It seems in such cases that the observers (especially students in elementary classes) are often confused when a few large granules or even chloroplasts, are seen moving apparently upstream. According to the present theory, the granules are merely moving along in the colorless stream of a granular cytoplasm. The granules apparently take no active part in the streaming process, but purely a passive one, as in the Foraminifera.

The idea of an active shearing force is not limited to filament streaming but may also be applied to the contraction that occurs in the posterior end of *Amoeba proteus*. In various other ameboid organisms and in leucocytes, it seems quite definite that contraction of the protoplasm does occur (Mast, 1926; Lewis, 1931; review, De Bruyn, 1947), but the supposed contraction of the protein molecules of the gel, as proposed by Goldacre and Lorch (1950) is merely a theory based on the old idea that muscle contracts by a folding or a spiralling of linearly arranged elongated protein molecules. Allen (1955) sucked protoplasm of *A. proteus* into capillary tubes and then observed two-way streaming, during which each stream behaved as a structural unit, which could became subdivided to form narrower streams, so small as to contain only a single row of granules. This seems similar to the two-way streaming in the reticulopodia of *Allogromia*. If this type of streaming can occur in the normal endoplasm of *Amoeba* it could be the physical basis of the contractile process. The possibility of such an explanation is mentioned by Noland (1957, quotation above).

Another alternative to the folding mechanism proposed by Goldacre and Lorch (1950) is the limited folding or sliding-folding mechanism suggested earlier by Frey-Wyssling (1948). According to this suggestion the sliding is caused by a wave of limited folding which passes along an elongated molecule. It is really an explanation, without experimental evidence, of how a sliding or creeping of one molecule along another might occur.

The contraction of the transparent pseudopodia of *Arcella* might also involve an active shearing rather than a molecular shortening, but on this point there is no evidence whatsoever.

The idea of an active shearing force can be applied to cyclosis whenever it occurs, *e.g.*, in *Nitella, Chara, Elodea, Paramecium, Vorticella,* etc. The only assumptions needed are that the moving material has a high viscosity and that the active force is exerted tangentially on its outer surface by the inner surface of the fixed cortical gel, or that the force is exerted from the moving high viscosity sol or gel on to the fixed cortex. Similar assumptions can also explain the rotatory movements of fragments of cells of *Nitella* and *Chara* described by Yatsuyangi (1953a, 1953b).

This possibility is well demonstrated in the work of Jarosch (1958) who has succeeded in isolating filaments from the protoplasm of *Toyellopsis* (Characeae). Jarosch has shown that these fibers are actively motile because they produce parallel displacement forces, that they have an affinity for small microsomes (*cf.*, granules of *Allogromia*), that they fuse into thick bundles, that they have the consistency of a gel, and that they possess elasticity. In brief, Jarosch has described in the filaments of *Toyellopsis* exactly the properties needed to explain streaming in *Allogromia*. In an earlier note he also mentioned the possibility of applying a similar theory to ciliary movement and to movement in axopodia (Jarosch, 1957).

In summary, it seems as if we can postulate two major types of protoplasmic movement in the Sarcodina, and possibly only these two types for all protoplasmic movements. These are:

1) Flow of a sol caused by differential pressure as a result of contraction of a partially surrounding gel, and

2) Movement of gel, which in *Allogromia* (and probably in plant hair cells) is in the form of paired filaments of gel which move by means of active shearing

forces, acting oppositely and longitudinally along the adjacent surfaces between the threads. In reticulopodia of most other Foraminifera the active force of the filaments, instead of acting on the other filament, might act against the stereoplasm, or, in axopodia, against the axoneme. In cyclosis the force from one layer of gel may act against that of another layer of thick sol or gel, or vice versa.

Furthermore, we have the possibility that the gel contraction that causes pressure flow may involve an active shearing mechanism as the contractile process of the gel.

4. *Taxonomic significance of the two types of protoplasmic streaming*

Certainly, if we consider only the Sarcodina as a group, we can state that we have both pressure flow and filament streaming as the two types of protoplasmic movement. If we ignore the untested possibility that both of these might be fundamentally the same, and consider them to be distinct, we are faced with the very interesting question of how they are distributed taxonomically. If this is a fundamental difference in the type of movement, perhaps all Sarcodina which have only filament streaming should be placed in a separate group from those which exhibit only pressure streaming, and those which have both should be placed in an intermediate group.

If this were done we would have to consider organisms with filopodia, reticulopodia, rhizopodia, and axopodia more closely related to each other than to those which have lobopodia only. This would necessitate changes in the well known separation of the Sarcodina into Rhizopodea and Actinopodea, and a re-assortment of the organisms placed in the rhizopodean order Proteomyxida, most of which have filament streaming. Such a thorough reorganization does not seem justified at present. However, the lines of demarcation between the current orders of the Sarcodina are so far from being satisfactory that a reclassification may well be contemplated in the future when more information becomes available.

5. *Nature of the reticulopodial surface*

The nature of the protoplasmic surface of reticulopodia has been the subject of comment by various investigators. It is commonly agreed that most but not all objects normally brought into contact with the pseudopodia will stick, and furthermore, *those that stick will be carried along in or on the protoplasmic stream.* Arnold (1953) mentioned this fact in regard to the food material used by *Allogromia.* In our experiments the insoluble dye and the glass particles stuck and were carried in both directions by the protoplasmic streams. Some particles do not stick tight enough to be engulfed. For instance, Sandon (1934) cites the fact that flagellates often stick to foraminiferan pseudopodia, are carried for a considerable distance, and then break loose and swim away. Sandon interprets this as evidence of a tough protective pellicle over the pseudopod. However, it could better be interpreted as evidence that the membrane is either thin and delicate as well as sticky, or even non-existent.

If one assumes that the streaming protoplasm is actually a thread of gel, then there is no need of assuming any membrane whatever in order to explain the mechanics of streaming. In fact, the principal reason for assuming the existence of a membrane is that to assume the opposite would be physiological heresy. It seems as if the assumption that a membrane does not exist is just as radical as the assumption of the existence of an active shearing force would have been a few years ago.

If we assume that a reticulopodial membrane does exist and that foreign particles which move with the flow are sticking to the membrane, then the membrane itself must move with the stream, as assumed by Sandon (1934). If so, then the portion of the membrane over the outgoing stream is moving oppositely from that over the ingoing stream, and the membrane, if it is to be considered a single membrane, must be sheared along two longitudinal lines, one on each side, where the circumferential margins of the oppositely moving streams are closest to each other. Therefore, along these two lines the membrane is continuously subject to longitudinal shearing and must be very highly labile; for all mechanical purposes such a membrane might as well not exist. The assumed membrane, then, as far as structure is concerned, becomes the membrane, not of the pseudopodium but of each protoplasmic stream, and it could well be merely the surface of the gel which makes up the moving protoplasmic thread.

On the other hand, if we assume that the foreign particles penetrate but are not completely covered by the membrane and stick to the moving gel thread, and that the membrane does not move, then the large particles, of a size, let us say, ten times the diameter of the pseudopodium, must split the membrane as it moves with the stream, and the membrane must be re-formed behind the particle. If we assume that these large particles upon contact with the gel are immediately covered by some sort of a membrane, then we must assume that the membrane is very rapidly formed at the anterior edge of the particle, and destroyed in the posterior edge of the particle, and this seems even more unlikly.

For these reasons it seems best to assume tentatively that the membrane of the small reticulopodia may be merely the surface precipitation membrane, of possibly merely the surface, of the moving thread of protoplasmic gel that constitutes the stream.

This tentative assumption, if made in addition to the theory of mechanism outlined above, results in perhaps the simplest overall concept of reticulopodia that is possible . . . merely two adjacent naked filaments of gel, or more exactly two parts of the same filament, pushing against each other longitudinally along their adjacent surfaces, with resultant two-way streaming. This concept may be oversimplified, but for the present there seems to be no reason for assuming without evidence the existence of any of the complicating structural considerations found in other material.

Summary

1. Protoplasmic streaming in the reticulopodia of *Allogromia laticollaris* is described. Streaming is always a two-directional movement of two threads of plasmagel which together with attached granules seem to make up the entire structure of the reticulopodia. There is no outer tube of gel, no central core of optically refractive material, and no space for an outer hyaline layer. This seems to be the simplest form of filament streaming known to exist.

2. It is proposed that the mechanism of filament streaming in *Allogromia* consists of active shearing or parallel displacement forces located between the adjacent surfaces of the two gel filaments, acting longitudinally and oppositely from one filament to the other so as to produce two-way streaming.

3. Possible applications of the theory of active shearing forces to protoplasmic movement in other materials are discussed.

4. It is suggested that in *Allogromia* the gel threads of the reticulopodia may not be covered by a typical cell membrane but by a surface precipitation membrane or that the membrane may be merely the surface of the gel filament itself.

5. The possible taxonomic significance of the existence of two major types of protoplasmic movements, namely, pressure flow and filament streaming, is discussed.

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OBSERVATIONS ON THE NUTRITION OF THE LAND PLANARIAN ORTHODEMUS TERRESTRIS (O. F. MÜLLER)[1]

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The Turbellaria as a class are carnivorous and previous investigations have shown that the range of prey available to these relatively simple animals has been greatly increased through the development of efficient feeding mechanisms in the form of progressive elaborations in the structure and use of the pharynx (Jennings, 1957). The Tricladida in particular, with the protrusible cylindrical type of plicate pharynx, are active and successful predators and the nutrition of the aquatic forms has already received much attention (Willier, Hyman and Rifenburgh, 1925; Kelley, 1931; Jennings, 1957). Little is known, however, of feeding and digestion in those triclads of terrestrial habit and hence a representative of this group, *Orthodemus terrestris* (O. F. Müller), has been examined to gain some measure of the influence of a land existence upon the typical pattern of triclad nutrition.

MATERIALS AND METHODS

Orthodemus terrestris occurs beneath limestone debris and fallen branches in the Fairburn, Malham and Settle districts of Yorkshire. Specimens collected throughout the year were presented in the laboratory with representatives of the fauna associated with them under natural conditions, and their food preferences and methods of capture and ingestion of the selected prey observed. The course of digestion was traced by histological examination of series of individuals previously starved to clear the gut and then killed at progressive intervals after being fed on either the natural food or test foods such as frog blood and boiled starch paste. After fixation in Susa at 30° C. sections were cut at 8 μ and stained with haematoxylin and eosin, Feulgen, benzidine, periodic acid-Schiff, Alcian blue and Lugol's iodine. Food reserves were studied after fixation in Flemming (for fat) and 90% alcohol containing 1% picric acid (for carbohydrates and proteins), sections of specimens fixed in the latter reagent being stained by the Best's carmine, P.A.S. and modified Millon methods.

OBSERVATIONS

The food and feeding methods

Orthodemus feeds mainly upon slugs (*Arion* sp.) and small earthworms. It will also attack small arthropods such as collembola, wood-lice, insect larvae and myriapods if these are injured or incapacitated in any way but normally they are too active for capture by the flatworm, which lacks any trapping or snaring devices. The mucus produced during locomotion quickly dries out and plays no part in the capture of food, unlike that of some aquatic triclads which persists about the habitat as sticky strands to entangle insect larvae and crustaceans. The prey appears to be found by chance and starved individuals show no awareness of the proximity of either damaged or intact animals until random movements bring them into direct contact.

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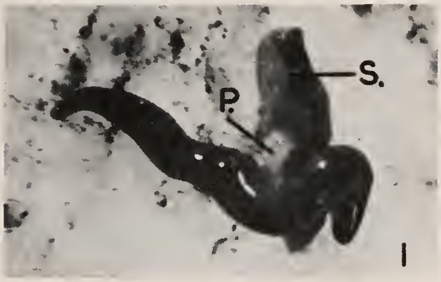
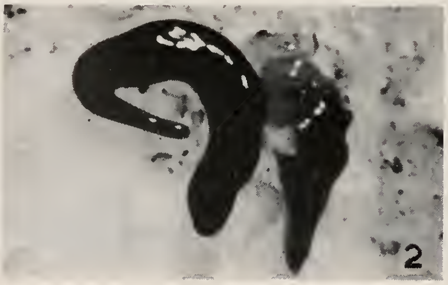
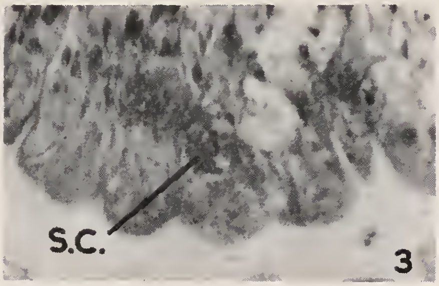
[1] New combination by Hyman (1954).

When an appropriately sized slug or earthworm is encountered the flatworm rapidly extends across the width of the prey until it can grip the substratum on each side and so pin the captured animal beneath the arched body. The grip on both prey and substratum is helped by copious secretions of mucus from the ventral surface and is so effective that prey rarely escape. Movement across the prey continues until the mouth, which lies ventrally approximately one-third of the body length from the posterior end, can be brought into contact with it. The muscular tubular pharynx is then protruded through the mouth and after moving rapidly over the surface of the prey is eventually thrust through the body wall (Fig. 1). When this occurs the flatworm changes position slightly to bring the mouth directly over the point of penetration to enable the pharynx to extend as fully as possible into the prey. The precise means of penetration could not be







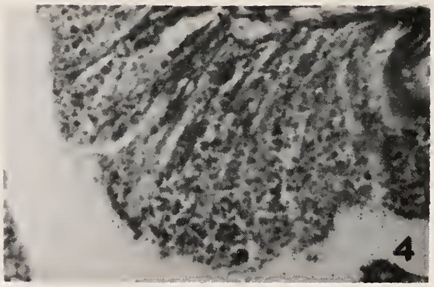


FIGURE 1. *Orthodemus* attacking a slug (S). The protruded pharynx (P) is penetrating the integument of the slug to withdraw the body contents. Magnification × 5.

FIGURE 2. Photographed 15 minutes after Figure 1. Feeding is complete and the flatworm is retracting the pharynx from the remnants of the slug. Magnification as in Figure 1.

FIGURE 3. A portion of the gastrodermis in *Orthodemus* showing a "sphere-cell" (S.C) surrounded by columnar cells. Haematoxylin and eosin. Scale: 1 cm. $= 20\ \mu$.

FIGURE 4. Gastrodermis of *Orthodemus*, 4 hours after a meal of starch paste, showing columnar cells loaded with phagocytosed material. Periodic acid-Schiff. Scale as in Figure 3.

ascertained, but it would appear to be purely mechanical, with the pharynx merely forcing its way inwards through the epidermis and musculature. Complete penetration is achieved within 30–60 seconds of first contact and there is no evidence to suggest the process is assisted by either regurgitation of solvent juices or the selection of external openings.

Once within the body cavity the pharynx moves around disorganzing the softer tissues to pass them back in a finely divided condition into the gut. The disruption of the tissues is rapid, and, like the penetration of the body wall, is mechanical with the pharynx acting as a simple suctorial tube extracting tissue fragments and body fluids. Withdrawal of the body contents continues until either only the collapsed and empty body wall is left, or the flatworm is replete, when the pharynx is retracted and the remnants of the prey abandoned (Fig. 2). Feeding lasts 10–20 minutes and during this period the flatworm lies in a very characteristic position across the prey, with the head region often raised and swinging slowly from side to side. In the early stages the food may be abandoned if the flatworm is disturbed or the incident light increased but later, when the pharynx is inserted and ingestion proceeding, the feeding individual is less susceptible to external stimuli and in the laboratory can often be manoeuvered into situations more suited to observation.

The laboratory stock of *Orthodemus* was sexually mature and produced cocoons between April and August. Up to six young, 3–4 mm. long, emerged from each cocoon within three weeks of laying and these fed in the same manner as the adults upon newly hatched or very young slugs. The latter were common in the habitat during the flatworm's breeding season and appear to form the staple diet of young *Orthodemus*, for isolated mucus cells and granules of a black pigment similar to that of the slug were often found amongst the gut contents of the young individuals fixed immediately after collection.

The structure of the gut and the course of digestion

The gut in *Orthodemus* has the typical triclad arrangement with numerous lateral caeca arising from each of the three main branches. The pharynx is of the cylindrical plicate type, and can be protruded through the mouth by simple muscular elongation.

The gastrodermis (Figs. 3 and 4) consists of a single layer of non-ciliated cells standing on a delicate basement membrane. Two types of cell occur. The larger and more numerous are columnar, 40–50 μ in height, with basal eosinophilous inclusions which apparently represent phagocytosed food particles, for they disappear rapidly during starvation. The second type is spherical or slightly pear-shaped, 25–30 μ high, and situated between the bases of the columnar cells in the ratio of approximately one to every ten of the latter. They contain eight to twelve proteinaceous spheres which stain heavily with haematoxylin and modified Millon method and disappear only slowly with starvation. These "sphere-cells" appear, therefore, to function as sites of protein storage—a conclusion supported by the absence of changes in appearance or staining reaction which can be correlated with digestive processes.

The presence of discrete particles within the columnar gut cells indicated that digestion in *Orthodemus* is intracellular and this was confirmed by an examination of individuals fixed at various intervals after observed feeds on slugs. Immediately after feeding the gut lumen is filled with a heterogeneous mass of cells, nuclei and muscle fragments already of suitable size for phagocytosis through the extreme disruption caused by the pharynx during ingestion. Phagocytosis begins as the first tissue fragments enter the gut and within fifteen minutes engulfed particles are found throughout the gastrodermis. They are contained at first in small vacuoles near the free distal border of the columnar cells, and phagocytosed muscle fragments and nuclei are clearly recognizable. With time, however, the fragments lose their identity and condense to homogeneous spheres which pass back deeper into the cells to disappear as digestion and absorption proceed. Four hours after feeding the columnar cells are loaded with phagocytosed material and they show a considerable increase in volume, with their walls becoming indistinct and the whole gastrodermis appearing almost syncytial. Complete digestion of a meal takes 12–24 hours, depending upon the amount of food taken, but any particles too big for phagocytosis, such as occasional large muscle fragments, are unaffected and remain unchanged in the gut lumen.

Experimental demonstration of the complete absence of intraluminar digestion was obtained by feeding frog blood and boiled starch paste. The former was readily taken by the flatworms but the starch appeared to be less palatable and had to be injected into a boiled portion of earthworm to ensure its ingestion. Fixation after blood feeding showed that many erythrocytes were ruptured during their passage through the pharynx, demonstrating its effective triturating action, and the corpuscle fragments, nuclei, and intact erythrocytes were quickly phagocytosed by the gut cells. Staining with the Feulgen and benzidine techniques showed progressive breakdown and disappearance of phagocytosed nuclei and haemoglobin, but in sharp contrast material in the lumen remained unchanged in appearance and staining capacity until it was either taken up by the cells or eventually expelled from the gut eight to twelve hours after feeding. Similar results were obtained after feeding with starch paste; staining with Lugol and P.A.S. showed digestion and absorption of starch within the columnar cells (Fig. 4), whilst that remaining in the lumen was quite unaltered.

It was not possible to determine the pH conditions of intracellular digestion owing to the limited number of specimens and the difficulty of administering food containing indicators.

The food reserves

Fat forms the principal food reserve in *Orthodemus* and the bulk is stored in the mesenchyme as large globules 15–20 μ in diameter, whilst smaller amounts are scattered as droplets 3–4 μ in diameter in the columnar cells of the gastrodermis.

As already stated, protein reserves are found within the so-called "sphere-cells" of the gastrodermis (Fig. 3) and in adult *Orthodemus* these show a marked seasonal variation. Thus in early spring the gastrodermis contains more "sphere-cells," each with large, dense and heavily staining spheres, but during the summer months when the gonads are mature and the flatworms producing cocoons the number of cells decreases and the spheres of those remaining shrink and stain only lightly. In the late summer, however, the cells start to increase in number and reach a maximum of one to every ten of the columnar cells by October or November.

Thus there is a build-up of reserve protein during the late summer which is rapidly depleted in the following breeding season.

There are no significant amounts of carbohydrate reserves. Staining with Best's carmine and P.A.S. reveals only very small amounts of glycogen which occur as tiny irregular granules scattered through the mesenchyme and columnar gut cells.

Discussion

It is evident from these observations that nutrition in *Orthodemus terrestris* differs very little from that described in the related aquatic triclads (Willier, Hyman and Rifenburgh, 1925; Kelley, 1931; Jennings, 1957). The typical triclad feeding mechanism, with the pharynx functioning as a suctorial tube which penetrates the prey to withdraw the body contents piecemeal, has apparently proved adequate to the needs of a terrestrial life and is retained unmodified. It allows the flatworm to deal effectively with slugs or earthworms which in the absence of devices for trapping more active animals appear to form the bulk of the diet. The failure of the mucous locomotory trail to persist and perform the secondary function of ensnaring the prey, as it does in aquatic triclads, is due perhaps to the terrestrial environment which although damp and humid does not prevent desiccation of the trail soon after its formation.

The retention of suctorial feeding, with extreme disruption of the food during ingestion, allows phagocytosis by the columnar cells to begin immediately food enters the gut. Consequently there has been no stimulus for the development of intraluminar digestion and the primitive condition of exclusively intracellular breakdown persists, exactly as in the aquatic triclads. A further similarity between the latter and *Orthodemus* is seen in the form and location of the food reserves, and particularly of protein stored in both cases in special "sphere-cells" in the gastrodermis.

It would appear, therefore, that the adoption of the terrestrial habit by *Orthodemus* has not necessitated any fundamental modification of the basic triclad methods of feeding and digestion. This is probably true of most other terrestrial triclads, for of the few existing accounts which mention nutrition, almost all describe or infer suctorial feeding upon earthworms, slugs and occasionally other invertebrates (Percival, 1925; Eastham, 1933; Johri, 1952; Froehlich, 1955; Pfitzner, 1958), and since this has such a profound effect upon the particle size of food entering the gut it is likely that it permits retention of purely intracellular digestion, as in *Orthodemus*. A few South American species, however, are reported to swallow their food whole (Froehlich, 1955) so that in these cases, unless preliminary breakup within the gut is achieved mechanically as in some rhabdocoels (Jennings, 1957), at least some degree of intraluminar digestion must occur.

I wish to thank Professor E. A. Spaul for his advice and encouragement during the course of this work.

Summary

1. The land planarian *Orthodemus terrestris* feeds principally upon small slugs and earthworms which are captured after chance encounter.

2. The typical triclad method of feeding, with the protruded cylindrical plicate

pharynx inserted into the prey to disrupt and withdraw the body contents, is used without modification.

3. Disintegration of the food during ingestion is so effective that the resultant particles are available for immediate phagocytosis by the gut cells and intraluminar digestion is absent.

4. The food reserves consist of fat stored in the mesenchyme and columnar gut cells, and protein stored in gastrodermal "sphere-cells." Protein reserves are depleted during the breeding season and replenished in the late summer and autumn.

5. It would appear that the basic triclad methods of feeding and digestive processes are quite adequate to the needs of terrestrial life and *Orthodemus* shows no particular adaptation to this so far as nutrition is concerned.

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THE CYTOCHROME SYSTEM IN MARINE LAMELLIBRANCH TISSUES[1]

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The occurrence of the cytochromes and cytochrome oxidase, very similar to the oxidase of mammals, has been proved in certain marine molluscs (Ball and Meyerhof, 1940; Humphrey, 1947; Ghiretti-Magaldi, Giuditta and Ghiretti, 1957). However, it is still obscure whether the cytochrome system acts as a terminal oxidation system in their intact tissues. The mere presence of the cytochromes or cytochrome oxidase in a cell does not indicate to what extent the normal respiration is mediated actually through the cytochrome system. Recently it was suggested that the cytochrome system may not play a major role in the respiratory system of the oyster mantle, although the tissue contains cytochrome oxidase (Jodrey and Wilbur, 1955).

The present investigation was undertaken to throw some light on the connection of the cytochromes with the respiration of intact tissues of marine lamellibranchs. A portion of this work has been preliminarily reported (Kawai, 1958).

MATERIALS AND METHODS

The respiratory studies were made at the Seto Marine Biological Laboratory during fall and early winter, and certain enzyme assays were carried out at the laboratory in Kyoto. Three species of marine lamellibranchs, the oyster, *Crassostrea gigas,* the pearl oyster, *Pinctada martensii* and the mussel, *Mytilus crassitesta,* were used as experimental materials. Specimens of the former two, each about two years old and about 11 and 6 cm. in shell height, respectively, were obtained from culture-farms in the vicinity of the Marine Laboratory, while *Mytilus,* about 8 cm. in shell height, was collected at a shore reef near the Laboratory.

Oxygen uptakes of intact tissues and extracts were measured at 25° C. in Warburg manometers, with vessels of about 9 ml. capacity. Respiratory measurements were carried out with several thin tissue pieces, 50 to 100 mg. in fresh weight, suspended in 1.5 ml. of sea water buffered at pH 8 with 0.03 *M* glycine (Robbie, 1946) or glycylglycine (Tyler and Horowitz, 1937). To absorb CO_2, 0.2 ml. of 0.5 *M* or 10% KOH and filter paper were placed in the center-well. In the cyanide experiments, NaCN was added to the main compartment and 0.2 ml. of KCN-KOH mixture (Robbie, 1946) was included in the center-well. Both glycine and glycylglycine of the concentration used had no effect on the respiration of the lamellibranch tissues. In the experiments of photo-reversibility of carbon monoxide inhibition, a 500-watt projector lamp was switched on at some distance away from the water-bath. A slight absorption by the KOH solution in the center-well was subtracted from each manometric reading.

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[1] Contributions from the Seto Marine Biological Laboratory, No. 334.

Absorption bands of cytochromes were observed with a low dispersion hand spectroscope and more accurate sites of the bands were measured with a Hilger wave-length spectrometer. The spectrophotometric studies of cytochrome oxidase were made with a Hitachi EPU-II spectrophotometer.

Carbon monoxide was prepared by decomposing formic acid with warm concentrated sulfuric acid. Cytochrome *c* was prepared from beef heart according to Keilin and Hartree (1945).

RESULTS

1. *Cytochrome spectra*

The absorption spectra of the reduced cytochromes were examined on intact thin tissues or breis, packed about 2 mm. thick, adding a small amount of solid sodium hydrosulphite. The characteristic absorption bands corresponding to cytochromes $a + a_3$, b and c, could be clearly observed in the lamellibranch heart at room temperature. Cooling the heart with liquid-air by the method of Keilin and Hartree (1949), the bands were very intensified, being slightly shifted towards the violet. The sites of these cytochrome bands were estimated with the Hilger spectrometer at room temperature as follows: $a\alpha + a_3\alpha$: 603; $b\alpha$: 562; $c\alpha$: 550; $b\beta + c\beta$: 520–530 mμ.

In other tissues, such as gill, mantle, adductor muscle, etc., only the band of cytochrome b could be detected at room temperature. However, a feeble band of cytochrome $a + a_3$ and a lesser band of c appeared at liquid-air temperature. Table I

TABLE I

Observations on the α-bands of reduced cytochromes in marine lamellibranch tissues at liquid-air temperature

Animal	Tissue	Relative intensity of the absorption*		
		Cyt. $a+a_3$	Cyt. b	Cyt. c
Crassostrea gigas	Heart	+++	+++	++++
	Gill	++	+++	+
	Mantle	+	++	+−
	Adductor muscle	+	++	+
	Digestive diverticula	+	++	+−
Pinctada martensii	Heart	++	+++	+++
	Gill	+	++	+−
	Mantle	+	++	+−
	Adductor muscle	++	++	+
	Gonad	+	++	+−
	Digestive diverticula	−	++	+−
Mytilus crassitesta	Gill	+	++	+−
	Mantle	+−	++	−

* Very strong absorption is shown by the sign +++ or ++++, while very feeble band is indicated by +−. The sign − shows the absence of cytochrome band.

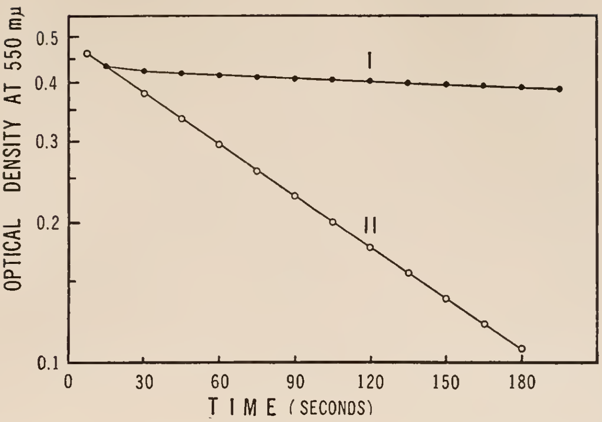

FIGURE 1. The oxidation of reduced cytochrome *c* by cytochrome oxidase from oyster gill. Reduced cytochrome *c* was prepared by reduction with a pinch of hydrosulphite and the excess hydrosulphite oxidized by shaking. Total volume 3 ml. Each cuvette contained final concentration of 2×10^{-5} *M* cytochrome *c*, 0.1 *M* phosphate buffer at pH 7.0 and 0.15 ml. of the extract. I contained 10^{-3} *M* cyanide; II, no cyanide. Reaction was followed at 25° C. The change of optical density was plotted on the logarithmic scale.

represents the results of these observations. The cytochromes are most abundant in the heart, where cytochrome *c* is predominant or equal to *b*. In other tissues, however, cytochrome *b* is apparently more dominant than $a + a_3$ or *c*. This fact forms a sharp contrast to the situation of the heart. Absence of the bands of $a + a_3$ or *c* in certain tissues may be due to their very low concentration. When a few drops of pyridine were added to the reduced tissues, a very intense pyridine hemochromogen band extending from about 550 to 560 mμ, with a mid-point at about 557 mμ, and a weak band lying about from 580 to 590 mμ were readily produced in all tissues examined. They are considered to be the absorption bands of pyridine derivatives of cytochromes *b* and *a* group, respectively.

2. *Cytochrome oxidase*

The enzyme activity was determined at 25° C. by two methods, *i.e.*, manometrically and spectrophotometrically. The extract for the enzyme study was prepared by homogenizing the excised tissue with a glass homogenizer in five parts of cold 1.24 *M* sucrose (isotonic with sea water) and squeezing through a thin cloth. The addition of an aliquot of the extract to a solution of reduced cytochrome *c* results in a rapid decrease in optical density at 550 mμ (Fig. 1). The activity of molluscan cytochrome oxidase is inhibited by about 94% in the presence of 10^{-3} *M* cyanide. The enzyme activity is also inhibited by carbon monoxide in the dark. Under the condition, 90% nitrogen + 10% oxygen in the control flask and 90% carbon monoxide + 10% oxygen in the experimental, the activity is inhibited by about 62% and the inhibition is completely eliminated by the illumination (Fig. 2).

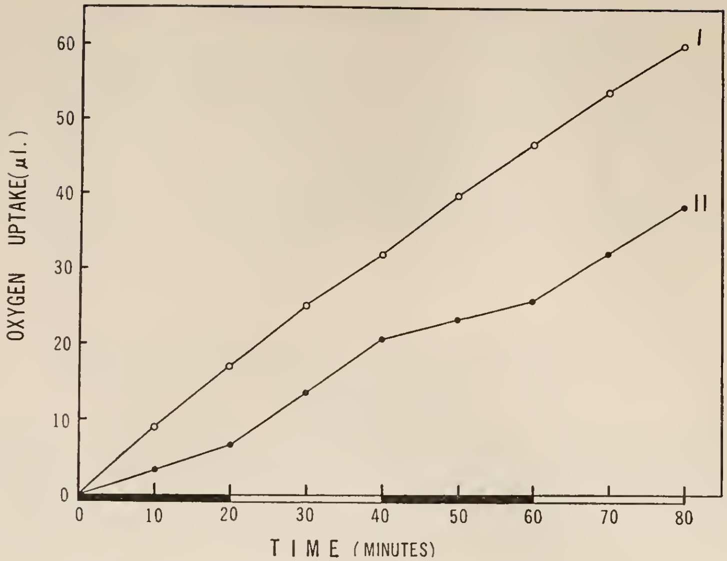

Figure 2. Effect of carbon monoxide on cytochrome oxidase from pearl oyster gill; 0.5 ml. extract in each Warburg flask. Final concentration: phosphate buffer at pH 7.0, 0.05 *M*; ascorbate, 0.01 *M*; cytochrome *c*, 2.2×10^{-5} *M*. Final volume 2.0 ml. I, in 90% N_2 + 10% O_2; II, in 90% CO + 10% O_2. Temperature 25° C. The black and white blocks under the base line show the periods of dark and light.

3. *Effect of carbon monoxide on the respiration of lamellibranch tissues*

Inhibition experiments were performed using the same gas mixture (CO/O_2 ratio of 9/1) as in the case of cytochrome oxidase. Controls with a gas mixture

Table II

Respiration of lamellibranch tissues in a gas mixture of 90% CO and 10% O_2

Animal, tissue	No. of determinations	O_2 uptake in control (μl./hr./100 mg. fresh wt.)	CO inhibition in darkness* (per cent)	Relative affinity constant**
Crassostrea gigas				
Heart	2	39	50	9.0
Gill	3	53	51	8.7
Mantle	2	29	53	8.0
Pinctada martensii				
Gill	4	48	47	10.1
Mantle	2	15	54	7.7
Digestive diverticula	1	27	46	10.6
Mytilus crassitesta				
Gill	2	26	52	8.3

* The complete elimination of CO inhibition by the illumination was observed in all tissues examined.

** Relative affinity constant of the tissue respiration for CO and O_2 was calculated by the Warburg equation. See text.

of nitrogen and oxygen (9/1) were also run, but there was no significant difference between oxygen uptakes in this control and in air.

Figure 3 shows the results of a typical experiment, obtained with the oyster mantle, indicating the presence of photo-reversibility of carbon monoxide inhibition. Nearly identical results, the inhibition of oxygen uptake in the CO gas mixture being about 50% in darkness and completely eliminated by light, were obtained in all tissues examined. These results are summarized in Table II. The relative affinity constants of the tissue respiration for carbon monoxide and oxygen were also calculated according to the equation of Warburg (1949); $K = n/1 - n \cdot pCO/pO_2$, where n is the fraction of oxygen consumption not inhibited, and pCO and pO_2 are, respectively, carbon monoxide and oxygen pressure. The values obtained, ranging from 7.7 to 10.6, are in good agreement with the average value of 8.2 reported for yeast cells in young cultures (Warburg, 1949). The stimulation effect of carbon monoxide on cell respiration, which has been found in certain marine eggs (Rothschild, 1949; Minganti, 1957; Rothschild and Tyler, 1958), was not observed in these lamellibranch tissues.

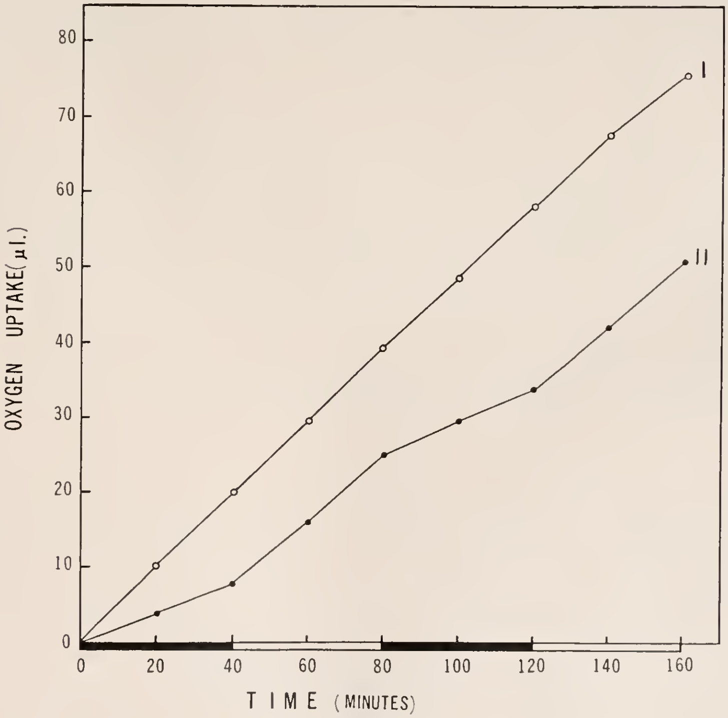

FIGURE 3. Effect of carbon monoxide on the respiration of oyster mantle. I, in 90% $N_2 + 10\% O_2$; II, in 90% $CO + 10\% O_2$. Each flask contained 100 mg. tissue pieces in 1.5 ml. of 0.03 *M* glycylglycine-buffered sea water. Temperature 25° C. The white blocks under the base line show the periods of illumination.

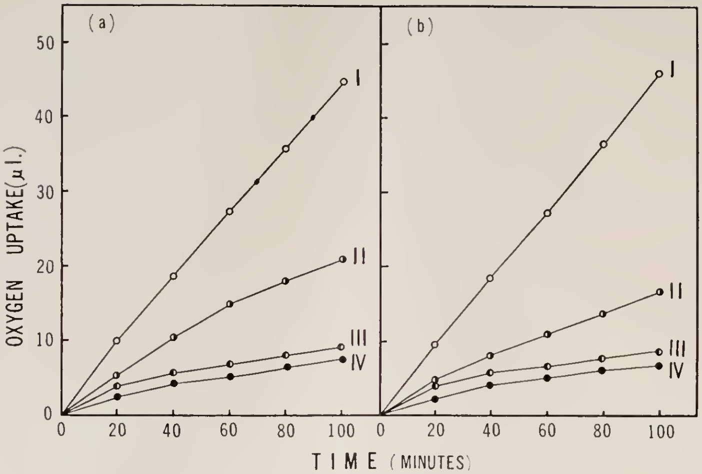

FIGURE 4. Effect of cyanide and methylene blue on the respiration of oyster tissues. (a) gill; (b) mantle. I, control; II, 10^{-3} M cyanide + 6×10^{-5} M methylene blue; III, 10^{-3} M cyanide + 10^{-5} M methylene blue; IV, 10^{-3} M cyanide. Each manometric flask contained 50 mg. of gill pieces or 100 mg. of mantle pieces in 1.5 ml. of sea water buffered with 0.03 M glycine. Manometric measurements were started twenty minutes after the tissues were immersed in each medium containing the reagents.

4. *Effect of cyanide on the respiration of oyster tissues*

The endogenous respiration of the oyster gill was depressed by 0.001 M cyanide to 15–20% of the control and the inhibition was partly restored to about 40% of the control in the presence of $6 \times 10^{-5}M$ methylene blue, or its nearly saturated solution in sea water; while a lower concentration of methylene blue, $1 \times 10^{-5}M$, was slightly effective to reverse the cyanide inhibition at an initial short period, but this effect completely disappeared within 40 to 60 minutes after the respiratory measurement was started (Fig. 4a). Very similar results, though the effect of $6 \times 10^{-5}M$ methylene blue was somewhat smaller, were also obtained in the cyanide inhibition of the oyster mantle respiration (Fig. 4b).

DISCUSSION

The marine lamellibranchs, *Crassostrea gigas*, *Pinctada martensii* and *Mytilus crassitesta*, used in this investigation have no oxygen carrier such as hemoglobin or hemocyanin in the blood. However, as reported here, their visceral cells possess a normal cytochrome system consisting of cytochromes *a*, *b*, *c* and a_3, *i.e.*, cytochrome oxidase. The absorption bands of cytochromes in their hearts being nearly equivalent to those in the cells of baker's yeast, it is inferred that the hearts probably contain as much cytochromes as yeast cells. Such high contents of cytochromes in the molluscan hearts, resembling mammalian hearts, may be concerned with their active movement. Although the contents of cytochromes are considerably low in other tissues, such as gill, mantle, adductor muscle, etc., there is apparently more cytochrome *b* than $a + a_3$ and *c*. This is very interesting when compared with the situation of the hearts, where cytochrome *c* is predominant or equal to *b*.

As the molluscan cytochrome oxidase shows photoreversibility in the carbon monoxide inhibition, it should contain iron atom, like the oxidase of mammals, in the active site. The relative affinity constant of the enzyme for carbon monoxide and oxygen, calculated from the equation of Warburg as described previously, is 5.5 under the condition employed. This value is very similar to that reported for cytochrome oxidase of sea urchin eggs (Krahl, Keltch, Neubeck and Clowes, 1941) and the spermatozoa of fresh-water mussels (Kawai and Higashi, 1959), but somewhat smaller than the value for mammalian cytochrome oxidase (Ball, Strittmatter and Cooper, 1951).

The presence of photoreversible inhibition of carbon monoxide in the respiration of lamellibranch tissues demonstrates that cytochrome oxidase acts as a terminal oxidase in these intact tissues. In a gas mixture of 90% carbon monoxide, the activity of molluscan cytochrome oxidase is inhibited by about 62% in the dark and the inhibition of the tissue respiration, though somewhat different in each tissue, is roughly 50%. Therefore, on the assumption that the oxidase *in vivo* is inhibited to the same extent in the extract, the respiration of intact tissues mediated through the cytochrome oxidase would be about 80% or more of the total respiration.

As is well known in many cells, autoxidizable redox dyes, exemplified by methylene blue, can restore the depressed respiration by cyanide. Such reversing effects, though not remarkable, could also be observed in the cyanide inhibition of oyster tissues using methylene blue (6×10^{-5} M) nearly saturated in sea water. According to Jodrey and Wilbur (1955), methylene blue, 1.4×10^{-5} M at the final concentration, was without appreciable effect in reversing the cyanide inhibition of the mantle respiration of the oyster (*Crassostrea virginica*). From this result they suggested that the cytochrome system may not play a major role in the oxidative metabolism of the oyster mantle. However, the ineffectiveness of methylene blue may be attributed to the matter of concentrations employed, because, in the present work, a lower concentration of methylene blue (1.0×10^{-5} M) was similarly ineffective to reverse the cyanide inhibition of the respiration of oyster tissues.

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SUMMARY

1. The cytochrome system of the lamellibranchs, *Crassostrea gigas*, *Pinctada martensii* and *Mytilus crassitesta*, has been studied in relation to the respiration of intact tissues.

2. The visceral cells possess a normal cytochrome system consisting of cytochromes *a*, *b*, *c*, and a_3, *i.e.*, cytochrome oxidase. The oxidase is strongly inhibited

by cyanide and carbon monoxide. The carbon monoxide inhibition of the enzyme is completely eliminated by light.

3. Cytochromes are most plentiful in the heart, where cytochrome *c* is predominant or equal to *b*, while *b* is apparently more predominant than $a + a_3$ or *c* in other tissues.

4. In a gas mixture of 90% CO and 10% O_2, the respiration of various tissues is inhibited by about 50% in the dark and the inhibition is completely eliminated by the illumination. Cyanide, 0.001 *M*, depresses the respiration of the oyster tissues to about 15–20% of the control, and the inhibition is partly reversed in the presence of methylene blue (6×10^{-5} *M*) nearly saturated in sea water.

5. It is concluded that about 80% or more of the total respiration of intact lamellibranch tissues proceeds through the cytochrome system.

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STUDIES ON THE MECHANISM OF PHOSPHATE ACCUMULATION BY SEA URCHIN EMBRYOS[1]

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It was shown by Needham and Needham (1930) that the developing larva of *Dendraster excentricus* increases its total phosphate content from fertilization to gastrulation. Since this initial discovery, phosphate accumulation by echinoderm eggs has been the object of a number of studies. The use of radioactive phosphorus in this analysis was introduced by Brooks (1943).

P^{32} uptake by unfertilized eggs is negligible. An exchange that does not result in an increase in the internal concentration of phosphate is thought to take place between the external and intracellular phosphate (Chambers and White, 1949, 1954; Brooks and Chambers, 1954). Lindberg (1949, 1950) considered that the surface of unfertilized eggs metabolized phosphate but that no intracellular penetration occurred. This surface metabolism involved incorporation of P^{32} into ATP.

The fertilized egg presents a radically different picture. Immediately following fertilization there is no change in P^{32} uptake, but within 6–30 minutes there is a noticeable increase. The rate of uptake increases to a maximum value and remains constant for as much as seven hours after fertilization. These initial events have been described for various species by Abelson (1947), Brooks and Chambers (1948, 1954), Whiteley (1949), and Chambers and White (1954). The maximum uptake rate by the fertilized eggs is as much as 160 times as great as the uptake by unfertilized eggs (Brooks and Chambers, 1948). The rate is not greatly affected by the accompanying decrease in P^{31} and P^{32} concentrations in the suspension medium within the limits 0.7 to 13 μM (Brooks and Chambers, 1954). Evidence that this uptake represents a real penetration is the fact that only 2 to 5% of the P^{32} activity is removed by continuous washing with sea water (Brooks and Chambers, 1948, 1954).

Radioactive phosphate is largely incorporated into the acid-soluble phosphate compounds (Abelson, 1947, 1948; Chambers, Whiteley, Chambers and Brooks, 1948; Chambers and White, 1949, 1954; Bolst and Whiteley, 1957). This is true both in the fertilized and unfertilized eggs. Among the acid-soluble components, the easily hydrolyzable phosphate compounds have the highest activity (Abelson, 1948; Lindberg, 1949, 1950; Chambers and White, 1949, 1954; Bolst and Whiteley, 1957).

Inhibition of uptake by fertilized eggs can be achieved by the use of low temperatures (Abelson, 1947; Villee and Villee, 1952). Metabolic inhibitors such as 4,5-dinitro-o-cresol (Abelson, 1947) and cyanide (Brooks and Chambers, 1948) also diminish uptake. These experiments have lead to the view that the developing embryo has an enzymatic mechanism that controls the penetration and consequent accumulation of phosphate. Lindberg (1949, 1950) has adduced evidence that in the fertilized egg the initial enzymatic reaction in the uptake involves incorporation of phosphate into adenosinetriphosphate. However, Chambers and Mende (1953) have determined that the primary penetration of phosphate through the plasma membrane still could be a matter of simple diffusion down an activity gradient inasmuch as they have found an extremely low concentration of free inorganic orthophosphate within the fertilized eggs of *Strongylocentrotus droebachiensis*. Chambers and White (1949) have found that the inorganic phosphate pool in *S. purpuratus* decreases quickly in response to fertilization. Agents that affect enzymatic activity might alter phosphate penetration by affecting the magnitude of the internal phosphate pool.

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In the present study the nature of penetration of phosphate into the fertilized egg of *Strongylocentrotus purpuratus* has been examined further. The experiments have largely involved an analysis of the effects of metabolic effectors (2,4-dinitrophenol, arsenate, ATP, and temperature) on the rate of uptake of P^{32} in fertilized eggs.

Methods and Materials

Experimental chamber. The basic experimental approach used in most of the experiments to be described was to measure continuously with a Geiger counter the accumulation of P^{32} by sea urchin eggs that were being perfused with sea water containing P^{32} as orthophosphate. In some experiments the perfusion fluid included various metabolic effectors. The lucite perfusion chamber was an improved version of that used by Chambers and Whiteley (Whiteley, 1949), and Chambers, White and Zeuthen (Zeuthen, 1951) (Fig. 1). Temperature was maintained constant by pumping water through the upper chamber. The eggs, which rested on the bottom of the lower chamber, were introduced through an opening in the side that was then closed by means of a lucite plug. The flow characteristics of the chamber were tested by perfusing it with a dye. The dye spread quickly and fairly homogeneously to all parts of the chamber and a flow of three to four ml./min. changed the sea water in the compartment to the extent of 90% in 1½ minutes as measured with a photo-cell. The volume of the chamber is 1.7 ml.

Radioactivity measurements. The chamber was placed on a lucite platform which in turn rested on the top of an end-window Geiger-Mueller tube; consequently the geometrical relationship between the tube and the chamber was always the same. The scaler was checked before and after each experiment against a calibrated standard. Several Geiger-Mueller end-window tubes were used during the course of the experiments. The thickness of their windows ranged from 2.3 to 3.1 mg./cm.^{2}. In the majority of experiments, corrections were made for the difference in sensitivity resulting from these differences in window thickness.

Conduct of experiments. Prior to entry into the egg chamber, the sea water was cooled by passage through coiled glass tubing in a constant temperature bath. In most experiments the temperature of the bath, and therefore in the chamber, was

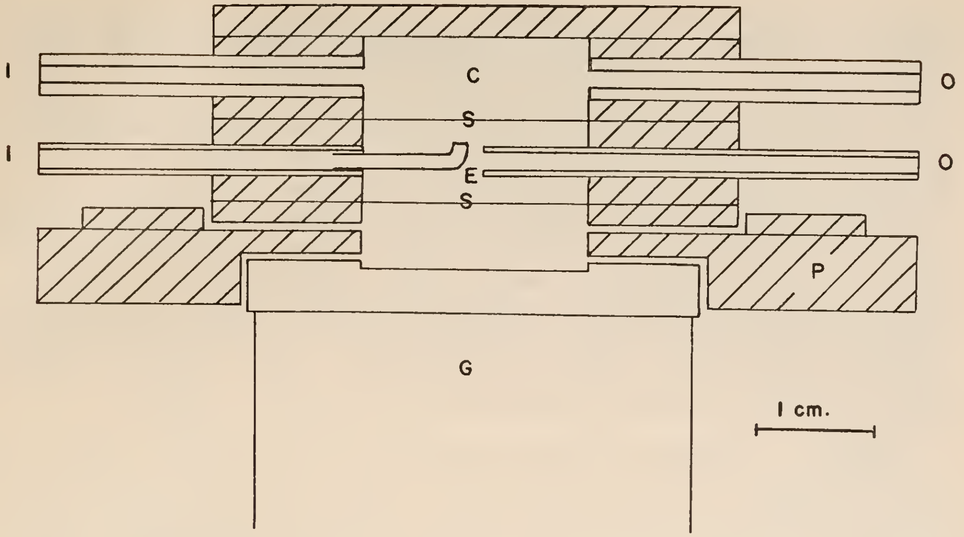

FIGURE 1. Perfusion chamber. c, cooling chamber; e, egg chamber; g, end-window Geiger-Mueller tube; i, inlet tube; o, outlet tube; p, platform; s, No. 1 coverslip.

18.0° C.; however for several experiments, the temperature was lowered to 8.0° C. Before each of these low temperature experiments, it was established with a thermocouple inserted into the chamber that the temperature of the water flowing through the egg chamber was 8.0° C.

At the beginning of each experiment, the eggs were perfused with sea water for fifteen minutes, while a background count for the experiments was obtained. Sea water containing P^{32} was then turned on and the activity in the chamber reached a new level with the unfertilized eggs. Fifty to sixty minutes after the start of the experiment, the eggs were fertilized. This was accomplished by injecting 0.1 to 0.2 cc. of a 10% sperm suspension in P^{32}-sea water into the chamber inlet tube with a hypodermic syringe. Fertilization and development of the eggs were observed with a Zeiss Opton stereoscopic microscope placed above the chamber. At the end of each experiment the eggs were removed from the chamber, washed, and allowed to develop further to check on their normality and on their recovery from the effects of any reagent that was being tested. After each experiment the chamber was rinsed alternately with concentrated HCl and NaOH and perfused with tap water and sea water in order to remove P^{32} adsorbed on the inner surfaces of the bottom compartment. Any radioactivity left in the chamber was accounted for by measuring the activity of the empty chamber just before each experiment.

In some experiments, eggs were activated parthenogenetically by the double treatment of Loeb (Just, 1939). The butyric acid and hypertonic sea water solutions were injected by means of a syringe into the egg chamber containing the unfertilized eggs. At the end of the treatment the eggs were perfused with sea water, followed by P^{32}-sea water as in the other experiments.

Materials. The eggs used were those of the sea urchin *Strongylocentrotus* *purpuratus* (Stimpson). The eggs from a single animal were used in each experiment. Shedding was induced either by injection of 4.25% KCl (Tyler, 1949), or by an electric shock of 30 volts, applied intermittently for several minutes (Harvey, 1952). Eggs were shed into filtered sea water and washed until 95% to 100% fertilization was obtained. In the majority of experiments the eggs were used within one to three hours after shedding. Sperm were collected in dry Syracuse watch glasses, and suspensions were made up just before use.

A 0.5% egg suspension was prepared and 1-ml. aliquots of this suspension were counted in a Sedwick-Rafter counting chamber. Based on this count, 15,000 eggs were transferred to the experimental chamber. During transfer and distribution of the eggs to the chamber 15 to 20% of the eggs were lost, so that in each experiment approximately 12,000 to 13,000 eggs were used. This number of eggs covered the bottom of the chamber in a single layer. Control eggs were cultured at 18.0° C. during the course of the experiment for comparison with those in the chamber.

Solutions. All perfusion solutions were made up in filtered sea water. P^{32} was obtained from the Abbott Laboratories as sodium phosphate in 0.9% NaCl at pH 6.5. P^{32}-sea water solutions were made to have an activity of 0.005 μc/ml. In all but two experiments the amount of carrier phosphate added was considerably less than the 31 to 62 μg/l. normally found in sea water in this area. The additional phosphate in these two experiments had no adverse effect on the eggs. One solution of P^{32}-sea water was prepared for each experiment, and from this P^{32}-solutions containing various test substances were made. All solutions were adjusted to pH 8.

RESULTS

Uptake of P^{32} by unfertilized, fertilized and parthenogenetically activated eggs

Experiments with unfertilized eggs confirm previous observations that there is practically no P^{32} accumulation at this time. A few minutes after the P^{32}-sea water first enters the egg chamber, the activity of the chamber reaches a level that

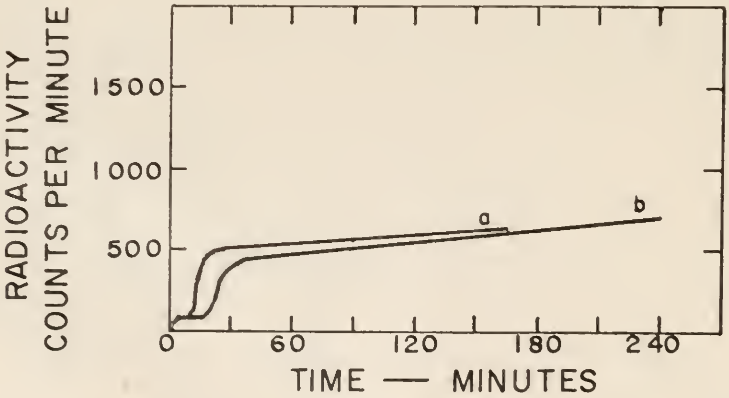

FIGURE 2. Uptake of P^{32} by unfertilized eggs of *Strongylocentrotus purpuratus* (Stimpson) and adsorption of P^{32} by the perfusion chamber. The initial low level of activity represents the background count. a, unfertilized eggs; b, empty chamber.

remains relatively constant for hours. During the course of several hours the activity may rise slightly, showing a small accumulation over the level in the sea water. The curves obtained with unfertilized eggs and with an empty chamber (Fig. 2) are very similar, from which it is concluded that the small rise is due to adsorption of P^{32} on the surfaces of the chamber and that no accumulation of P^{32} occurs in the unfertilized eggs. This conclusion is strengthened by the results of several experiments in which it was found that the rate of increase in activity in the chamber is not affected by varying the number of unfertilized eggs from 5,000 to 20,000.

In contrast to the unfertilized eggs, fertilized eggs accumulate P^{32} so that the activity in the chamber soon greatly exceeds that of the P^{32}-sea water. An experiment that typifies the course of this uptake is given in Figure 3. Two other

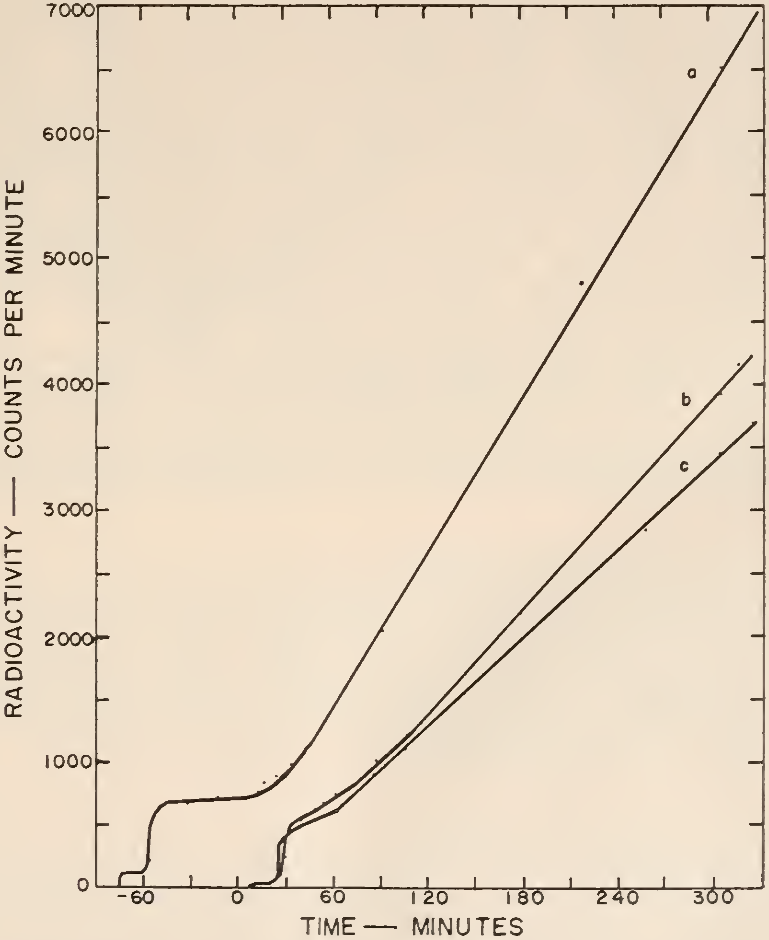

FIGURE 3. Uptake of P^{32} by sperm activated and artificially activated eggs. The initial low level of activity represents the background count. Activation was at zero time. a, sperm activated eggs; b and c, artificially activated eggs.

experiments are similar in all essential respects. This accumulation commences after a short lag period that is quite variable, ranging in 24 experiments, from 7 to 30 minutes with an average of 18. The maximum rate of uptake is not established until approximately 40 minutes after fertilization. This again is variable, ranging from 22 to 60 minutes in 14 experiments. While an explanation of these variabilities is not at hand, there seems to be no correlation between the length of the lag period and the length of time the eggs have been out of the ovary, which varied from 1 to 5.75 hours in 24 comparable experiments. It is probable that the cause resides in inherent differences in the gametes of different animals. Once the maximum rate of uptake is established, it remains constant. This constant rate of uptake was observed in experiments that continued for five hours after fertilization.

The characteristic uptake pattern of the fertilized eggs could be dependent to some degree on the penetration of the sperm, or it could be inherent solely in the potentialities of the eggs. To answer this question, eggs were activated parthenogenetically and the uptake of P^{32} followed. As is shown in Figure 3, such eggs exhibit a P^{32} accumulation comparable to that of fertilized eggs. The onset of phosphate accumulation is also preceded by a lag period. Uptake was initiated by both a single treatment with butyric acid and a double treatment of butyric acid and hypertonic sea water. The rate of P^{32} uptake in these experiments was approximately 75% of the fertilized egg rate. This difference was probably due to the activation of only 60–70% of the eggs in the chamber, as measured by membrane elevation. Moreover, none of the activated eggs cleaved normally. It seems probable that under optimum conditions, with activation approaching 100%, the uptake would approach very closely that of the fertilized eggs.

The time of onset and the magnitude of phosphate uptake appear to be potentialities of the egg, then, and are independent of the sperm. The increase in uptake does not begin until well after the visible cortical events of membrane elevation have occurred. It is of interest also that the well-known increase in respiration associated with fertilization begins earlier than the phosphate uptake and is not clearly associated with it.

Effect of low temperature on P^{32} uptake

Two experiments were carried out in which the temperature was initially 8.0° C. followed by an increase to 18.0° C. in the middle of the experiment. At 8.0° C. there was a comparatively low rate of P^{32} uptake, which was readily stimulated when the temperature was raised to 18.0° C. (Fig. 4). From these experiments a Q_{10} of 2 and 2.3 was calculated comparable to the value of 2 obtained by Villee and Villee (1952) with *Arbacia punctulata.*

Effect of 2,4-dinitrophenol on P^{32} uptake

While the establishment of the high uptake rate does not coincide with the establishment of the high respiratory rate, and the pattern of uptake during cleavage does not resemble the exponentially increasing respiratory rate, the effect of temperature still suggests that phosphate uptake may be related to energy metabolism.

2,4-dinitrophenol (DNP) is a metabolic inhibitor known to interfere especially

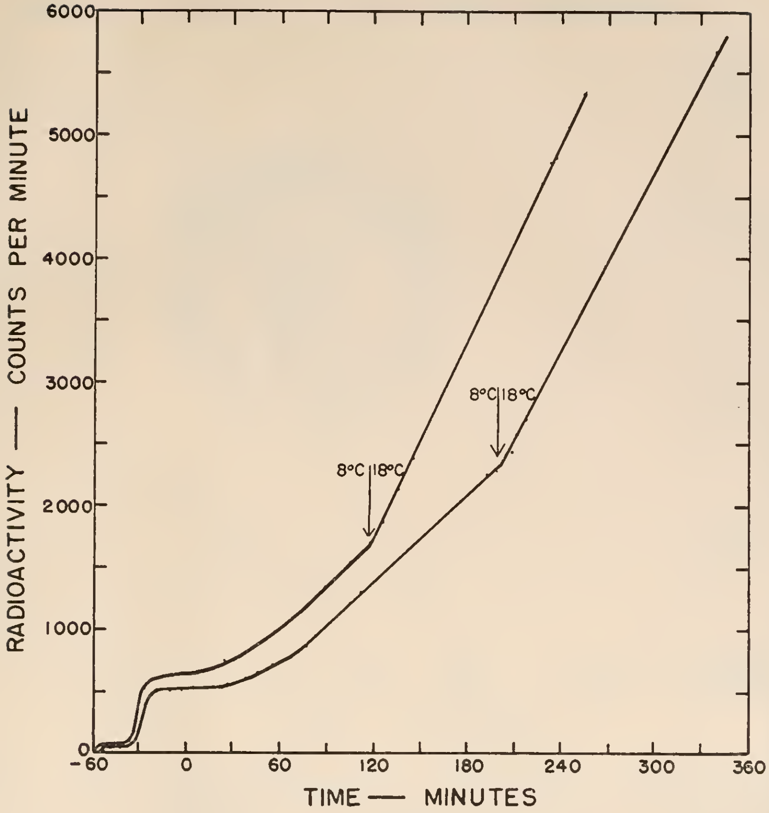

FIGURE 4. Effect of temperature on P^{32} uptake. The initial low level of activity represents the background count. Insemination was at zero time. Arrows indicate a change in the temperature of the perfusion solution.

with aerobic phosphorus metabolism with the result that it uncouples phosphorylations from oxidations (Loomis and Lipmann, 1948) and so in turn interferes with energy-requiring processes (Simon, 1953). Inhibition of phosphate uptake has been reported by Abelson (1947) for dinitrocresol, another substituted phenol. However, in some unpublished experiments, Whiteley had observed that when DNP was added some time after fertilization, there was no inhibition of phosphate uptake. A detailed investigation of this point has shown that the time when DNP is applied has a direct bearing on its effect on P^{32} uptake. In these experiments DNP in a concentration of 10^{-4} *M* in sea water was introduced into the perfusion chamber at various times before and after fertilization, and the effect on the rate of P^{32} uptake was measured. This concentration will reversibly inhibit cleavage. When DNP is applied before fertilization or any time within the first thirty minutes following fertilization, there is a marked inhibition of P^{32} accumulation, as may be seen in Figure 5. After thirty minutes, the effect of DNP decreases until, when it is added at sixty minutes after fertilization, it has no effect on P^{32}

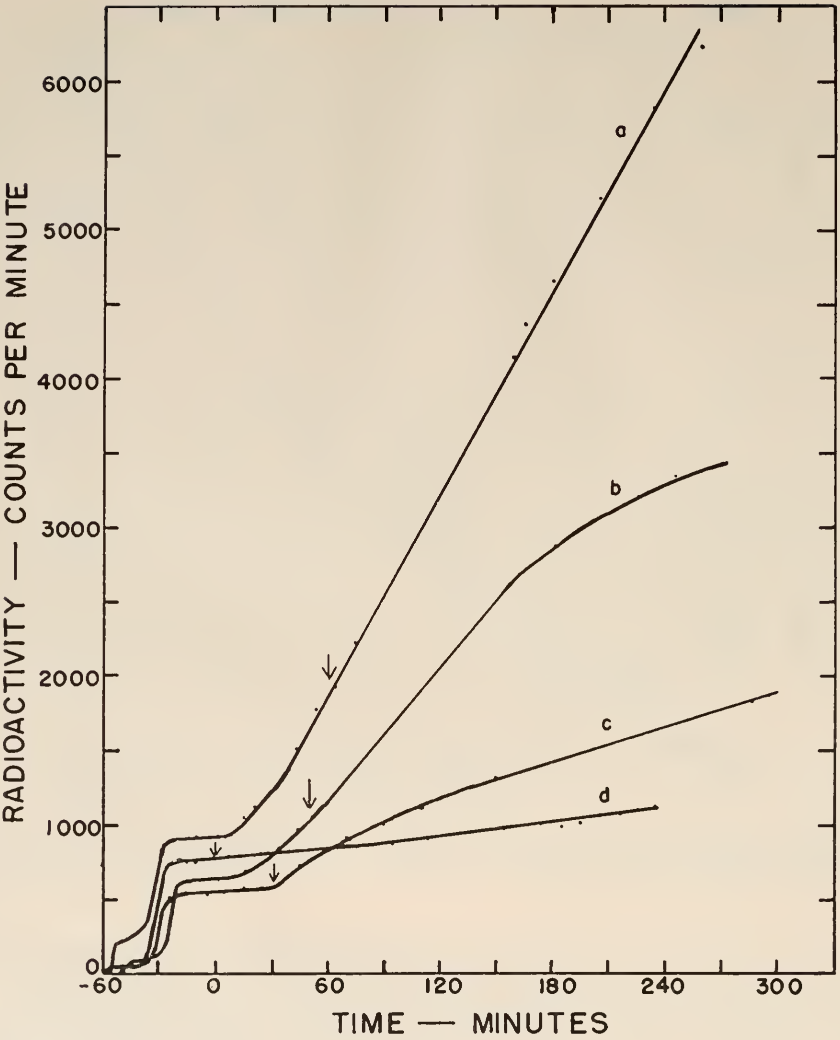

FIGURE 5. Effect of 10^{-4} *M* DNP on P^{32} uptake. The initial low level of activity represents the background count. Insemination was at zero time. Arrows indicate the addition of DNP to the perfusion chamber. a, DNP added at 60 min.; b, DNP added at 50 min.; c, DNP added at 30 min.; d, DNP added at insemination.

accumulation. If the rate of P^{32} uptake is plotted against the time after fertilization when DNP is applied, the time course of the inhibition can be clearly seen (Fig. 6). The maximum inhibition is associated with the first 30 minutes following fertilization. The degree of inhibition at 40 minutes is variable. This variability may be correlated with the length of the lag period and the onset of the maximum uptake rates: if the latter is not established until after the application of DNP at 40 minutes, the inhibition seems to be appreciable; if the maximum rate of uptake

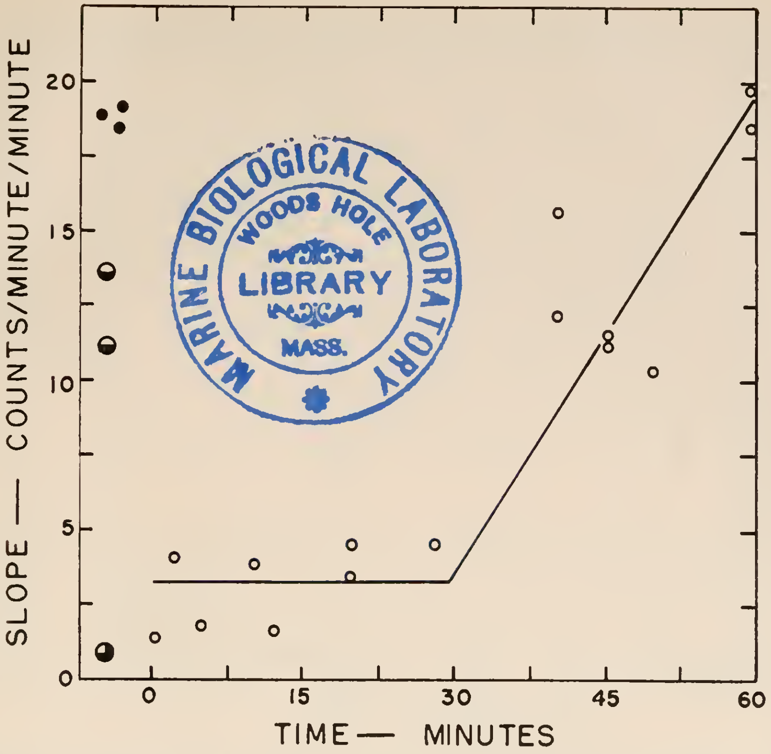

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FIGURE 6. Effect of 10^{-4} *M* DNP on the rate of accumulation of P^{32} when the DNP is added at various times after insemination. Ordinate—average slope from insemination to the end of the experiment. Abscissa—time after insemination when the DNP was added. Open circles: fertilized eggs and DNP; solid circles: fertilized eggs; half-open circles: artificially activated eggs; quarter-open circles: unfertilized eggs.

has been established by 40 minutes, the inhibition is considerably reduced. Once P^{32} uptake has been firmly established, as at 60 minutes after fertilization, DNP has no effect on the accumulation, even in experiments continued for three hours after the application of the DNP, and despite inhibition of cleavage. Fertilized eggs are most sensitive to DNP, as far as P^{32} uptake is concerned, at a time when virtually no P^{32} uptake has been established.

Effect of adenosinetriphosphate on dinitrophenol inhibition

Kriszat and Runnström (1951), Barnett (1953) and Kriszat (1954) have reported that ATP will partially reverse the cleavage block due to DNP. In the light of these results it was thought that ATP might overcome the inhibitory effect on P^{32} uptake produced by DNP applied during the lag period. The two experiments of Table I were done to test this possibility. To conserve ATP the perfusion chamber was not used in these experiments. In each experiment four equal aliquots containing about ten thousand fertilized eggs were placed in four small flasks. At four to six minutes after insemination the flasks received P^{32} at a final concentration of 0.01 μc/ml. and either sea water, DNP, ATP, or DNP and ATP. Final concentrations of DNP and ATP (Sigma crystalline disodium adenosine 5′-triphosphate) were 10^{-4} *M* in each instance. In Experiment 1 the final volumes were 100 ml., and in Experiment 2, 50 ml. The pH of the cultures was 7.8 to 8.0 and they were maintained with stirring at 15.5° C. Orthophosphate concentrations in the cultures varied from 2.1 to 3.8 μM. Two hundred and seventy minutes after adding DNP and ATP in the first experiment and 90 minutes in the second, the embryos were gently centrifuged and washed three times with sea water. The control eggs removed less than 12% of the available P^{32} from the culture solutions. It is clear that while DNP is markedly inhibitory when added so soon after fertilization, 10^{-4} *M* ATP does not relieve the DNP inhibition. ATP itself, in these and one other experiment, depresses uptake 15 to 28%. In both experiments cleavage was completely inhibited by the DNP, even in the presence of ATP. In the longer experiment only 5 to 10% of the embryos cleaved when subsequently placed in sea water at the end of the experiment. Recovery was 90 to 95% in the shorter experiment.

The negative results could mean that DNP damage is not repaired by direct addition of ATP, or that insufficient ATP penetrated to activate the energy-dependent reactions blocked in the presence of DNP. The permeability of developing eggs to ATP was tested directly in several experiments in which the concentration of ATP in sea water bathing eggs was measured during the first 8 or 9 hours of development. In these experiments recently inseminated eggs were suspended in sea water containing 5×10^{-5} *M* ATP (Sigma, crystalline Na_2ATP) at pH 8.0 and at 15.1° C. The egg concentrations were about 1% by volume or 10,000 eggs/ml. Aliquots of the suspensions were collected immediately and at intervals until the ninth hour of development, and were assayed for the concentrations of adenine-containing compounds (absorbance at 260 mμ), inorganic phosphate, and acid-labile phosphate (phosphate hydrolyzed in 10 minutes in 1 N HCl at 100° C.). Controls consisted of sea water with ATP, but no eggs, and sea water with eggs but no ATP.

If ATP were absorbed to or penetrated into the eggs, the absorbance of ultraviolet light and the acid-labile phosphate would diminish proportionately. If ATP were hydrolyzed to ADP or AMP by surface-located enzymes without penetration of the adenine moiety, the acid-labile phosphate would decrease, but not the ultraviolet absorbance. In different experiments the removal of adenine-containing components by the eggs varied from 0–2.34×10^{-4} μmoles/10,000 eggs/hr., and the maximum observed change in ATP that could be ascribed to a

Table I

$P^{32}O_4$ cpm/aliquot

Sample	Exp. 1 (in DNP for 270 min.)	Exp. 2 (in DNP for 90 min.)
Control eggs	6583	6642
Eggs in 10^{-4}*M* ATP	4758	5362
Eggs in 10^{-4}*M* DNP	17	42
Eggs in 10^{-4}*M* ATP and 10^{-4}*M* ATP	14	50

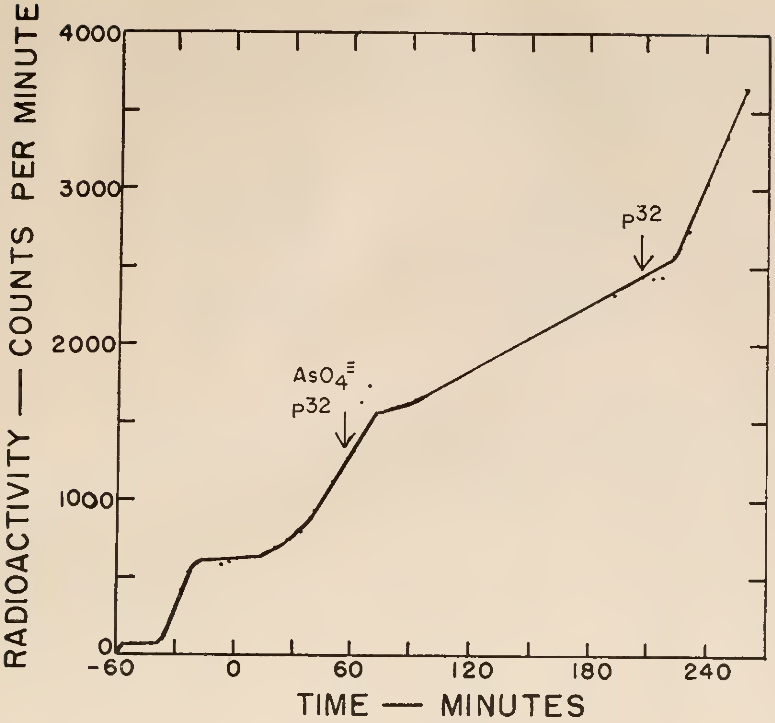

FIGURE 7. Effect of $10^{-4} M$ arsenate on P^{32} uptake. The initial low level of activity represents the background count. Insemination was at zero time. Arrows indicate a change in the perfusion solution.

surface-located ATPase was 7.9×10^{-4} μmoles/10,000 eggs/hr. Both these figures are at the limits of the techniques, and indicate practically no change in the ATP in the sea water around the eggs. Therefore, the failure of ATP to relieve the DNP inhibition of P^{32} uptake during the lag phase may be due to insufficient penetration of the added ATP.

Effect of arsenate on P^{32} uptake

Arsenate, because of its structural similarity, is a competitive inhibitor of phosphate in a number of enzymatic reactions.

As shown in Figure 7, a concentration of $10^{-4} M$ sodium arsenate markedly inhibits P^{32} uptake. Unlike DNP, this inhibition occurs whenever arsenate is applied from fertilization to 3½ hours after fertilization. The inhibition is completely reversible upon removal (Fig. 7, Table II). A decrease in the rate of P^{32} accumulation sets in within 1 to 5 minutes after the arsenate has first entered the chamber, and recovery from this inhibition takes place within a comparable period of time upon removal of the inhibitor. When arsenate is applied at fertilization, a limited uptake commences after a lag period that is within the range of the normal lag period, for example, 26 minutes in one experiment. Therefore,

TABLE II

Effect of 10^{-4} M arsenate on P^{32} uptake

Exp. No.	Interval after fertilization	Perfusion solution	Rate of P^{32} uptake, counts/min./min.
1	0–88 min.	AsO_4, P^{32}-sea water	6.0
	89–143 min.	P^{32}-sea water	25.0
	144–200 min.	AsO_4, P^{32}-sea water	7.0
2	0–54 min.	P^{32}-sea water	20.3
	55–204 min.	AsO_4, P^{32}-sea water	6.9
	205–260 min.	P^{32}-sea water	28.0

arsenate does not seem to increase the length of the lag period, but markedly and reversibly suppresses penetration during the accumulation phase.

It has been tentatively concluded by Yĉas (1950) that arsenate does not penetrate the eggs of the echinoid *Lytechinus pictus*. This conclusion was based on experiments that showed that arsenate had no effect on respiration or on cleavage. It was assumed that, if it had penetrated, there would have been an interference with oxidative phosphorylation, and consequently respiration and cleavage would have been affected. An experiment was set up to confirm these observations and extend them to *S. purpuratus*. Small numbers of fertilized eggs were placed in finger bowls of sea water containing sodium arsenate at various concentrations five minutes after insemination, and ninety-five minutes after insemination. The time required for these embryos to attain the early cleavages, and the normality of subsequent development were then determined (Table III). A concentration of arsenate of 10^{-4} *M* has an almost imperceptible effect on early cleavages and shows a clear retardation of development only at early blastula stages. More serious retardations set in after 8 hours in 10^{-4} *M* arsenate, leading to death in an early blastula condition at 28 hours, when controls are late blastulae. One hundred-fold stronger concentrations delay the first three cleavages by only about 20%, although development beyond 8 hours involves increasing abnormalities, with death at 22 hours. In experiments not included in Table III, 10^{-3} *M* arsenate showed retardations only a little greater than 10^{-4} *M*. Only small quantitative differences were caused by adding the arsenate at 5 minutes as compared with 95 minutes after insemination. It is doubtful that arsenate penetrates the eggs at an appreciable rate during cleavage. Therefore, the effects of arsenate on phosphate uptake are interpreted as evidence for a surface location of the penetration mechanism.

DISCUSSION

The evidence, taken as a whole, suggests the view that the rapid penetration of phosphate into sea urchin embryos is an enzymatically controlled transport. The reaction has a temperature coefficient of 2 to 2.3, which is compatible with this possibility though not, by itself, conclusive (Danielli, 1952). It is inhibited by arsenate, a competitive analogue of phosphate. Furthermore, other investigators

TABLE III

Effect of arsenate on development of embryos of S. purpuratus. Solutions were at pH 8.0 and 11.4° C., and had an orthophosphate concentration of about 2×10^{-6} *M. Arsenate was added at 5 min. (column A) or 95 min. (column B) after insemination*

Time after insemination	Controls	10^{-4} M AsO_4		10^{-2} M AsO_4	
		A	B	A	B
1 hr. 50 min.	50% 2-cell	50% 2-cell			
1 55			50% 2-cell		50% 2-cell
2 7				50% 2-cell	
2 55	50% 4-cell		50% 4-cell		
3 3		50% 4-cell			
3 7					50% 4-cell
3 25				50% 4-cell	
4 7	50% 8-cell				
4 20		50% 8-cell	50% 8-cell		50% 8-cell
4 55				50% 8-cell	
7 20	32-cell	50% 32-cell		16-cell, many abnormal	
11 35	early blastulae	very early blastulae		abnormal late cleavage	
22 35	rotating blastulae	early blastulae		dead very early blastuale	
23 20	hatching	early blastulae		—	
28 27	late blastulae	dead		—	

have found that the uptake is independent of the external phosphate concentration over a wide range (Brooks and Chambers, 1954; Chambers and White, 1954).

That the mechanism of phosphate entry is surface-located seems most probable from the results of the arsenate experiments. Arsenate must penetrate only very slowly, if at all, into the early cleavage stages. This follows from the observations of Yĉas (1950) and those reported here, that arsenate, even at the concentration of 10^{-2} M, shows little effect on cleavage of these eggs, and retardations occur only after some hours. The conclusion is strengthened further by Yĉas' finding that 0.05 M arsenate does not inhibit respiration of fertilized and cleaving eggs of *Lytechinus pictus*. In marked contrast the strong inhibition exerted by 10^{-4} M arsenate on phosphate uptake is evident in a very few minutes. The arsenate must have its effect at the surface by competition with phosphate for a surface-located transport mechanism, and the reaction is probably not directly linked to respiratory metabolism.

The specific reaction by which phosphate enters the embryo is not definitely elucidated by these experiments, but certain possibilities are suggested by the arsenate experiments. Arsenate is known to be a competitive analogue of phosphate, and therefore will substitute for it in enzymatic reactions. The resulting arsenate compound is usually unstable and is hydrolyzed instantly. The term arsenolysis has been applied to the action of arsenate in splitting organic compounds (Doudoroff, Barker and Hassid, 1947).

Arsenate has been shown to inhibit the enhancement of P^{32} uptake by adenosine in mammalian red blood cells (Prankerd and Altman, 1954). In this system arsenolysis is presumed to result in a decrease in glyceraldehyde-3-phosphate. The conversion of glyceraldehyde-3-phosphate to 1-3-diphosphoglyceric acid near the surface of the cell is proposed as the mechanism of phosphate entry into the human red blood cell (Prankerd, 1956). The same mechanism could be operative in the fertilized eggs, although arsenate would also inhibit other phosphate esterifications.

The time of establishment of the mechanism is within the first 40 to 50 minutes, varying with eggs from different animals. Whether its appearance as a functional system is during the lag period of 7 to 30 minutes, or whether the subsequent period of increasing activity represents the time of establishment is not answered by these experiments. The experiments with parthenogenesis show clearly that the establishment of the mechanism is not dependent on the sperm, nor on the existence of a normal cleavage mechanism.

There remains to be considered the relation between the egg's metabolism and the transport mechanism. The presence of DNP during the first 30 minutes after fertilization prevents very markedly the later uptake of phosphate. It is assumed that DNP is exerting its characteristic uncoupling action and is consequently inhibiting the formation of high-energy phosphate by aerobic oxidations at this time. However, it appears that there is enough energy for this process at 60 minutes after fertilization despite the presence of DNP. At this time also, DNP is presumably having its effect on oxidative phosphorylation since cleavage is reversibly blocked. A correlation between oxidative phosphorylation and cleavage has been shown by Clowes, Keltch, Strittmatter and Walters (1950), whose experiments demonstrate that the concentration of a substituted phenol that will block oxidative phosphorylation in cell-free particulate systems of *Arbacia punctulata* will also inhibit cleavage in the intact egg. The conclusion drawn from this similarity in effective concentrations is that DNP inhibits cleavage by interfering with high-energy phosphate production.

Two interpretations of the effects of DNP on P^{32} uptake present themselves. According to one, the initial period may be sensitive to DNP because aerobic phosphate bond energy is needed for the synthesis of the enzymes of the transport mechanism as is the case with adaptive enzyme synthesis (Monod, 1944), or perhaps for the spatial rearrangement of the pre-formed system. The later period may be insensitive to DNP because the maintenance and operation of the mechanism requires smaller amounts of aerobic phosphate bond energy.

An alternative idea is based on a suggestion from a paper of Siekevitz and Potter (1953). It may be that the energy source for the establishment of a phosphate entry mechanism is different from that for its maintenance and operation. In experiments with rat liver mitochondria they concluded that the ATP generated within the mitochondria diffused out and mingled very slowly with that generated by glycolysis outside of the mitochondria. Consequently, there may be a separation in the functions of the ATP formed in these two locations. Synthetic reactions within the mitochondria would preferentially utilize ATP generated locally, while an energy-requiring reaction outside of the mitochondria would be served by ATP produced by glycolysis externally. It may be that the establishment of a phosphate transport mechanism involves enzyme synthesis that is favored by ATP formed within the mitochondria. DNP could inhibit such a process by its action on the tricarboxylic acid cycle, as the enzymes for this cycle are associated with the mitochondria. Once the mechanism is established it might be maintained by high-energy phosphate resulting from glycolysis. DNP presumably does not inhibit the formation of high-energy phosphate by this means, although this has not been fully investigated (Simon, 1953). Glycolysis has been implicated in phosphate uptake by yeast (Rothstein, 1954) and by the mammalian red blood cell (Prankerd, 1956).

The negative results of the experiments testing the efficacy of externally applied ATP to overcome the early DNP inhibition are explained by the finding that ATP neither penetrates the fertilized eggs of this species, nor is adsorbed to their surface, nor is hydrolyzed at their surface in amounts detectable by the rather sensitive methods used. This failure of ATP to be efficacious is in contrast to its effect on cleavage inhibition by DNP reported by Kriszat and Runnström (1951), Barnett (1953), and Kriszat (1954). It is doubtful that the tiny amounts of energy that could have been available to the eggs, judging from the present data, would be sufficient to overcome the inhibitions as found by these investigators. If the eggs of their studies were not appreciably more permeable to ATP, one wonders if the effects could be ascribed to other substances in their ATP preparations.

According to the results of Bolst and Whiteley (1957) the rate of penetration of phosphate increases rapidly for 35 hours in the embryos of *S. purpuratus*. This is in accord with the present findings that the transport system is surface-located because, during the development to the gastrula, the number of cells, and therefore the surface area of the embryos, increases through cleavage, and it is reasonable to suppose that the newly formed surface would possess the transport mechanism.

SUMMARY

1. The accumulation of phosphate by the eggs and embryos of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson) was analyzed by determining the action of various metabolic effectors on the uptake of P^{32} from sea water flowing at a constant rate over the eggs in a special perfusion chamber.

2. The rate of uptake of P^{32} by unfertilized eggs is nearly zero. The rate for the first 7 to 30 minutes after fertilization (lag phase) is also nearly zero, but increases rapidly during the next 20 to 30 minutes (augmentation phase), and becomes maximal 22 to 60 minutes after fertilization (accumulation phase) at a level many times that of the unfertilized eggs.

3. Artificial parthenogenesis, by either the single or double treatment, results in the same pattern and magnitude of uptake as does fertilization, even in the absence of cleavage.

4. Phosphate accumulation is markedly inhibited by 10^{-4} M 2,4-dinitrophenol if this agent is added during the lag phase, moderately inhibited if added during the augmentation phase, but is unaffected if added during the accumulation phase.

5. Addition of 10^{-4} M ATP simultaneously with dinitrophenol early in the lag phase does not alleviate the inhibition caused by the latter.

6. ATP neither penetrates into cleaving eggs nor is hydrolyzed by an ATPase at their surface.

7. 10^{-4} M arsenate markedly inhibits P^{32} uptake at all times after fertilization.

8. Arsenate does not materially retard early development of these sea urchin eggs indicating that it does not penetrate into them.

9. P^{32} uptake has a temperature coefficient of 2 to 2.3 during the accumulation phase.

10. The evidence resulting from the use of these effectors indicates that P^{32} uptake in sea urchin embryos is enzymatically controlled and that the enzymatic mechanism is located on the cell surface. The period immediately following fertilization is believed to be a time when the uptake mechanism is being established. This process appears to be dependent on phosphate bond energy, the production of which is DNP-sensitive. During the accumulation phase it is suggested that the energy requirements for the operation and maintenance of this mechanism are quantitatively much smaller, or are satisfied by phosphate bond energy the production of which is DNP-insensitive. Possible reasons for this difference in sensitivity are discussed.

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SOME EFFECTS OF TEMPERATURE ON DEVELOPMENT IN THE SEA URCHIN ALLOCENTROTUS FRAGILIS

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The deep water sea urchin *Allocentrotus fragilis* occurs offshore at Pacific Grove at depths of 80 to 100 fathoms. The temperature of this environment is fairly constant at 8° C. During the past two years it has been possible, at this Station, to study the embryological development of this form under laboratory conditions. Normal development of the fertilized eggs proceeded at 7°–15° C. Lower temperatures were not tried. There was some variation at the upper limit, the eggs of some females not developing normally above 14° C. while others yielded normal embryos at 16° and 17° C. At ordinary room temperature (20° C.), however, cytoplasmic cleavage is not normal if it occurs at all. A study of this effect may throw some light on the behavior of cytoplasm and nucleus in cell division.

In order to determine whether the nucleus might be responsible for the failure of cleavage at 20° C., the viability of the nucleus was tested by putting it to develop in the hardy cytoplasm of *Strongylocentrotus purpuratus*. The eggs of this purple sea urchin develop normally at the higher temperature, and when fertilized with the sperm of *Allocentrotus fragilis* they segment and develop into normal plutei at 20° C. Similarly the eggs of the sand dollar *Dendraster excentricus* fertilized with the sperm of *Allocentrotus fragilis* segment normally at the tempo of *Dendraster*. Such hybridized eggs develop into plutei, which, in their form, show the cross to be a true one. Hence the male nucleus has taken part in development. Thus, while the cytoplasm of *Allocentrotus* is inactivated at a temperature of 20° C., the nucleus of this species, if given a proper medium, functions normally.

Visual proof of the fact that this nucleus also functions in the egg of its own species even at 20° C. is shown by putting fertilized eggs of *Allocentrotus* to develop at 20° C. Here the nuclei undergo regular division while the cytoplasm remains apparently inert, *i.e.*, undivided (Figs. 1 and 2). Now these eggs had formed normal fertilization membranes at the outset before being put at the higher temperature. Therefore there is no reason to suppose that here we are dealing with a Sugawara (1943) effect in which failure of the fertilization membrane to lift results in constriction of the egg to its original volume with the results that cleavage of the egg is inhibited but nuclear division proceeds. Eventually the eggs with which we are dealing, after many nuclear divisions, disintegrate. This experiment gives further proof that in the early stages the nucleus is more resistant to higher temperatures than its cytoplasm and carries out its divisions independently of the cytoplasm.

In the later stages of development, when the cytoplasm has become comminuted into the thousand odd cells of blastula and gastrula, the spatially relative situation of nucleus and cytoplasm is quite different. If now the advanced larvae of

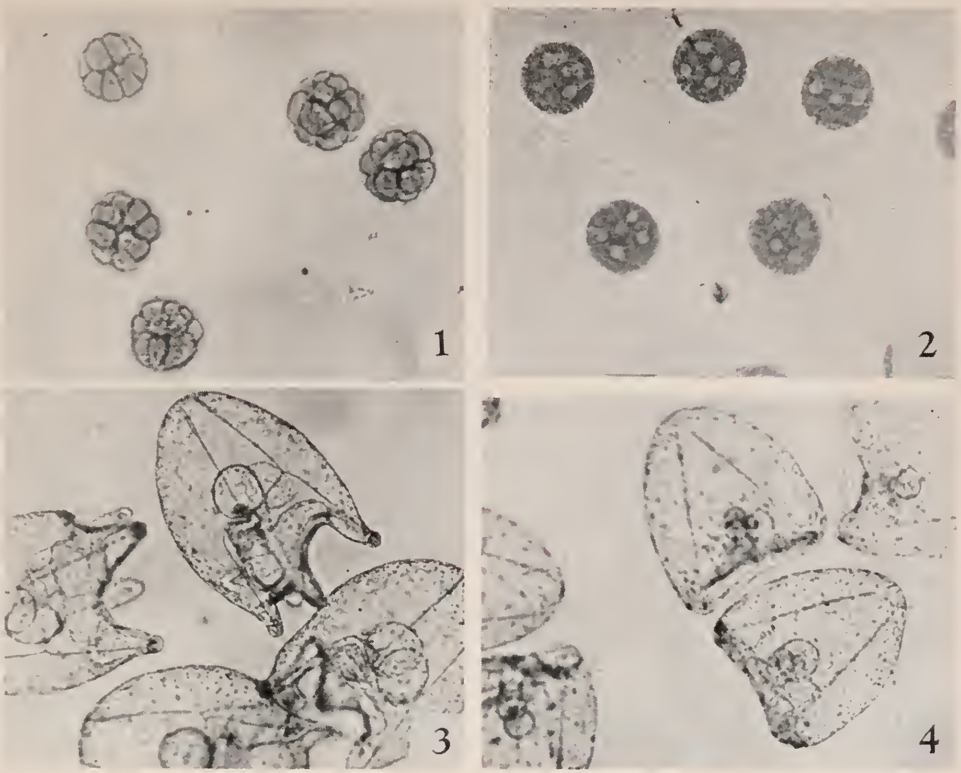

FIGURE 1. Normal 8–16 cell embryos at 15° C. FIGURE 2. Same at 20° C. FIGURE 3. Five-day pluteus developed at 15° C. FIGURE 4. Five-day plutei in which the temperature was changed to 20° C. after development to gastrulae at 15° C. Magnification 95 ×.

Allocentrotus were transferred from the cold room where they had developed to a warm room with a temperature of approximately 20° C., it was found that the higher temperature no longer exercised a lethal effect on the cytoplasm, but that such larvae reached the pluteus stage (Figs. 3 and 4). These plutei were smaller and less elaborate than the normal ones developed at the low temperature.

DISCUSSION

Generally speaking the problem of cleavage has been attacked from the point of view of colloidal behavior, the relation of cytaster formation to the constitution of the cell which is the substance of the egg. Cleavage is a process initiated by the entry of the sperm into the egg and the formation of the membrane. In artificial parthenogenesis the formation of the membrane is sufficient to initiate the development of cytasters and subsequent cleavage of the egg. A second factor has been suggested by Swann (1952) who postulates the active agents in cleavage to be catalytic substances released from the chromosomes of the sperm nucleus. These he has termed "structural agents."

In the foregoing experiments we have evidence that the differences in the interaction of nucleus and cytoplasm described depend on the nuclear-plasma relation. Thus in the early stages of development the mass of the cytoplasm compared to that of the nuclei is relatively enormous and the distance of the nucleus from the periphery, it seems reasonable to suppose, may be too great for the cytoplasm to be significantly affected by substances diffusing from the nucleus. It should be noted that the nucleus in the first division is dominated in the tempo of cleavage by the cytoplasm. This has been clearly shown in the case of *Dendraster* which has a cleavage time for the first division of 55 minutes at 20° C. If the experiment be made of enucleating the egg of *Dendraster* and then fertilizing it with the sperm of *Strongylocentrotus,* the cleavage time of which is approximately 100 minutes, the subsequent division of the nucleus and cytoplasm of the experimental egg takes place in the time characteristic of *Dendraster, i.e.,* of the cytoplasm and not of the nucleus. Thus the normally slow nucleus is forced by the cytoplasm to divide in a little more than half the time normal to it (Moore, 1933).

As to the difference in their reaction to the higher temperature on the part of the fertilized eggs of *Allocentrotus* contrasted with that of blastulae and gastrulae, it may be suggested that, in the latter, the nuclei are in such intimate contact with the cytoplasm that they confer some of their hardiness on it and are able to do this because of the close association of the two phases. Such an effect becomes understandable if we accept Swann's hypothesis as to the part played by the nucleus in the cleavage of the egg. Using Chambers' (1951) demonstration in the amoeba that the nucleus dynamically affects the edge of the cell next to it, Swann has suggested that the origin of the furrow in the first division is caused by a cleavage substance which diffuses from the chromosomes of the sperm nucleus to the periphery of the egg and initiates cleavage. In the present experiments Swann's hypothesis seems not to apply in the early stages of cleavage. In later stages, however, this hypothesis may give a reasonable explanation of the division of cells in advanced larvae at higher temperatures. Thus, while in the early stages of cleavage the cytoplasm dominates the formation of the furrow and the tempo of cleavage, the situation is altered later where successive divisions of the cytoplasm have brought the chromosomes into intimate relations with the cytoplasmic units. This would give play to the diffusion of cleavage substances from the chromosomes to the periphery as Swann has postulated. Since the effect of the higher temperature on the further development of blastulae and gastrulae is to make the resulting plutei defective, it seems reasonable to suggest that at the time of change only a part of the synthesis of structural elements has been completed and that the higher temperature to which they were subsequently exposed has inhibited the completion of structural processes essential to the form of the normal pluteus.

SUMMARY

1. The eggs of the deep water sea urchin *Allocentrotus fragilis* develop normally to plutei under laboratory conditions at 7°–15° C.

2. At the higher temperature of 20° C. cytoplasmic division fails but the nuclei show characteristic mitotic figures.

3. The sperm nucleus of *Allocentrotus fragilis* functions normally at higher

temperatures in the eggs of *Strongylocentrotus purpuratus* and *Dendraster excentricus*.

4. Blastulae and gastrulae of *Allocentrotus fragilis* brought to a temperature of 20° C., which is lethal for the eggs and early division stages, develop into plutei of reduced size.

5. It is suggested that in the advanced larvae hardiness to the higher temperature is the result of the intimate association of nucleus and cytoplasm in the minute cells, and the synthesis of structural elements and processes at the lower temperature.

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A REVISION IN THE SPHAEROMID GENUS GNORIMOSPHAEROMA MENZIES (CRUSTACEA: ISOPODA) ON THE BASIS OF MORPHOLOGICAL, PHYSIOLOGICAL AND ECOLOGICAL STUDIES ON TWO OF ITS "SUBSPECIES"

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During a recent study into the physiology of osmoregulation in sphaeromid isopods (Riegel, 1959), an interesting problem was brought to light concerning the taxonomy of *Gnorimosphaeroma oregonensis* (Dana, 1852). Menzies (1954a) split the species into two subspecies, *lutea* and *oregonensis*. *G. o. oregonensis* was described as a typical intertidal bay form, inhabiting the undersides of rocks in waters whose salinity approached that of normal sea water. *G. o. lutea* was described as a creek or pond dweller confined to waters of low salinities and often associated with mud and vegetation. Differences in osmoregulatory ability and ecological requirements, as well as morphological differences, lead the writer to doubt the validity of Menzies' subspecies.

Riegel (1959) performed experiments on specimens from fresh-water, estuarine and bay populations of *Gnorimosphaeroma oregonensis* to test their osmoregulatory abilities. He found that specimens of the bay form [= *G. o. oregonensis* (Dana)] could neither regulate their body fluid concentration within viable limits nor survive for longer than a few days in fresh water. Specimens of the estuarine and fresh-water forms of *G. oregonensis* (= *G. o. lutea* Menzies 1954) were able to regulate their body fluid concentrations within viable limits and survive for over three weeks in salinities ranging from fresh water to 125 per cent sea water. Specimens of the fresh-water form, when in 50 per cent sea water or less, could maintain significantly higher body fluid concentrations than could specimens of the estuarine form. All forms of *G. oregonensis* were found to regulate hyper-osmotically in dilute media and hypo-osmotically in salinities just below or above normal sea water.

The present paper reports the results of experiments and observations designed to clarify the taxonomic position of the "subspecies" of *Gnorimosphaeroma oregonensis* described by Menzies (1954a). The differences which prompted the unofficial separation of that species into three habitat groups (*i.e.*, estuarine, fresh-water and bay forms) in a previous paper (Riegel, 1959) are no longer under primary consideration, so to avoid confusion, Menzies' subspecific names will be used throughout the balance of this paper.

METHODS

Experiments were conducted to determine the effect of salinity on the morphology of *Gnorimosphaeroma oregonensis lutea* and *G. o. oregonensis*. One hundred young of each "subspecies," newly emerged from the brood pouch, were placed in separate finger bowls containing rocks and normal sea water. The normal sea water in which the young *G. o. oregonensis* were placed was diluted over a period of two weeks to ten per cent sea water. Controls consisting of newly-emerged young of both "subspecies" were kept in normal habitat water, but otherwise under identical conditions. The young isopods were fed slices of frozen shrimp occasionally, and the water was changed weekly.

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In another experiment, more than two hundred ovigerous females (plus over a hundred males and immature individuals) of *Gnorimosphaeroma oregonensis lutea* were placed in a large plastic tub containing normal sea water and otherwise simulating the natural habitat as closely as possible. The "brood pouches" of the females contained eggs and embryos in all stages of development. The animals were fed slices of frozen shrimp occasionally. Samples of twenty young were removed from the tub each week (for three months) and examined for possible morphological differences created by the high salinity. This experiment was done in order to subject specimens of *G. o. lutea* to high salinity as early in their embryological development as possible, in case the effect of salinity (as measured in the first experiment, described above) is no longer exerted on the morphology of the animals after they leave the brood pouch.

To ascertain to what extent (if any) populations of *Gnorimosphaeroma oregonensis lutea* and *G. o. oregonensis* intergrade ecologically, the writer made extensive surveys in San Francisco Bay, Tomales Bay, and coastal inlets from Half Moon Bay, San Mateo County, to Stillwater Cove (near Fort Ross), Sonoma County, California.

As the result of handling large numbers of *Gnorimosphaeroma oregonensis lutea* and *G. o. oregonensis,* the writer observed that the body in the latter form seemed relatively broader than in the former form. Therefore, measurements of length and width were taken of random samples of the two "subspecies" from various localities and salinities. From these measurements, length/width ratios were calculated and analyzed statistically.

RESULTS

The newly-emerged young of *Gnorimosphaeroma oregonensis lutea* and *G. o. oregonensis* died slowly over a six-week period. That their deaths were not due to salinity alone is indicated by the fact that the controls also died slowly. However, during the six-week period, some growth was detected (both by increase in size and the presence of cast-off exoskeletons), but no changes in the morphology of the animals were seen.

The young removed from the plastic tub in which were kept the ovigerous female *Gnorimosphaeroma oregonensis lutea* all showed the typical morphology of that "subspecies." Of particular significance was the fact that young which underwent their entire development in normal sea water showed no change from the typical morphological configuration of *G. o. lutea.* These were young hatched from eggs which were in the "brood pouches" of the females when the females were originally placed in normal sea water. At the end of the experiment, no ovigerous females were found in the plastic tub and many of the young were half grown (3–5 mm. long).

The results of surveys designed to determine the degree of ecological intergradation between *Gnorimosphaeroma oregonensis lutea* and *G. o. oregonensis* were as follows: *G. o. oregonensis* was found in only three locations, Stillwater Cove, Point San Quentin in San Francisco Bay, and Tomales Bay State Park in Tomales Bay. *G. o. lutea* was found in Pilarcitos Creek draining into Half Moon Bay, several creeks draining into Tomales Bay, and in the Napa River and several creeks draining into the northern end of San Francisco Bay. In only one location did the two "subspecies" occur in the same general area, which was at Tomales Bay State Park. At the other locations cited above for *G. o. lutea,* conditions were probably not suitable for *G. o. oregonensis,* since the substrate was either muddy or sandy and lacked the rocks and loose rubble which characterize the habitats from which the writer has collected the latter form. At Tomales Bay State Park, where the two "subspecies" occurred together, there was a sharp break in their respective habitats. *G. o. lutea* was found in a small creek among the vegetation and under rocks. Little more than 50 feet away, but in the intertidal area of the rocky beach, *G. o. oregonensis* was collected from under rocks and among the loose rubble. The only known barrier present was the very dilute salinity of the creek water (fresh to taste), which would probably prevent *G. o. oregonensis* from entering the creek. Since *G. o. lutea* is capable of living in salinities approaching normal sea water, there appeared to be no salinity barrier to its colonization of the intertidal area. However, no specimens referable to *G. o. lutea* were found in the *G. o. oregonensis* habitat, and no specimens of *G. o. oregonensis* were found in the *G. o. lutea* habitat. At Stillwater Cove, *G. o. oregonensis* were collected in the loose rubble and under stones intertidally. There was a small stream running into the cove, which appeared suitable for habitation by *G. o. lutea,* but that form was not found there.

From measurements of the body length and width of random samples of ten specimens of each of the two "subspecies," the following ratios were found. The length/width ratio of the body of *Gnorimosphaeroma oregonensis oregonensis* averages 1.64 ± 0.021, with a range of 1.50 to 1.75. The length/width ratio of the body of *G. o. lutea* averages 1.84 ± 0.018, with a range of 1.70 to 1.96. The mean differences were found to be highly significant statistically ($t = 7.29$). Subsequent checks of specimens of both "subspecies" from different localities and salinities made since the above measurements were taken bear out the decided separation in the range of length/width ratios between *G. o. oregonensis* and *G. o. lutea.*

DISCUSSION

Osmoregulatory ability in relation to ecology

In general, the osmoregulatory abilities of the various experimental groups reported in a previous paper (Riegel, 1959) and summarized in the present paper, agree well with their habitat and distribution according to salinity tolerance.

The fresh-water form of *Gnorimosphaeroma oregonensis lutea* is a good osmoregulator, which would be expected of a successful invader of fresh water. One barrier to complete adaptation to fresh water, namely, the ability to reproduce and rear young in that medium, possibly has been circumvented by this form. The brood pouches of most isopods are formed by flaps (oöstegites) projecting medially from the bases of four pairs of thoracic legs. The developing eggs and embryos are held between the overlapping flaps and the sternum. However, in the Sphaeromidae, the eggs and embryos are carried in various types of internal "brood pouches" (Menzies, 1954b) which in *G. oregonensis* (*sensu lato*) are separated from the body fluid only by a thin and presumably permeable membrane. Thus, by regulating the body fluids osmotically, the sphaeromid probably can regulate the osmotic environment of the eggs and embryos. Young *G. o. lutea* which hatched out in the laboratory survived for several weeks in fresh water. Whether reproduction in fresh water is dependent upon the internal "brood pouch" of *G. o. lutea* or due to osmoregulatory ability or osmotic resistance of its embryos and eggs is not known.

Populations of *Gnorimosphaeroma oregonensis lutea* living in brackish water must adjust to salinity variation of at least two types: (1) Daily fluctuations in salinity of relatively short duration during the tidal cycles, and (2) seasonal fluctuations due to rainfall and runoff from melting snow. Measurements of habitat salinity and body fluid concentration changes during a portion of a tidal cycle (Riegel, 1959) showed the pattern of salinity fluctuations to which an estuarine population of *G. o. lutea* adapted. Between a period from low to high tide, the salinity of the habitat varied from fresh water (0.27% sea water) to 65 per cent sea water. During the same period, the body fluid concentrations of the resident *G. o. lutea* varied from 50 per cent sea water (in fresh water) to 70 per cent sea water (in 65% sea water). Another population of the "subspecies" which lived in a pond situation away from tidal salinity influences had to adjust to salinities as high as 60 per cent sea water during the late summer and fall, and as low as ten per cent sea water during the winter and spring periods of rain and heavy runoff from melting snow.

The internal "brood pouch" of *Gnorimosphaeroma oregonensis* living in brackish water is of possible value as a "buffering" mechanism to prevent damage to eggs and embryos by extreme fluctuations in the salinity of the habitat, especially in the lower salinity ranges.

Gnorimosphaeroma oregonensis oregonensis has never been collected from salinities approaching fresh water. The lowest salinity recorded in its habitat was about 12 per cent sea water, which occurred during a period of particularly heavy rains; presumably, this form is capable of surviving for short periods in such low salinities. Further, it is improbable that the animals must ever endure prolonged exposure to low salinity. During low tide, they are always found down in the coarse rubble on the beach. During conditions of low salinity in the general habitat, it is probable that in their microhabitat the salinities are higher due to water trapped by spaces in the rubble, leaching of residual salts from the rubble and evaporation.

Osmoregulation and other factors in relation to systematics

As mentioned previously, Menzies (1954a) split *Gnorimosphaeroma oregonensis* into two subspecies, *G. o. oregonensis* and *G. o. lutea.* However, because of differences in their osmoregulatory physiology and habitat preference, the validity of Menzies' subspecies is questioned by the writer.

The biological species is defined by Mayr *et al.* (1953, p. 313) as consisting of "groups of actually (or potentially) interbreeding natural populations which are reproductively isolated from other such groups." Mayr *et al.* define a subspecies (p. 314) as "a geographically defined aggregate of local populations which differs taxonomically from other such subdivisions of the species." Let us analyze *Gnorimosphaeroma oregonensis* in the light of the above concepts.

Gnorimosphaeroma oregonensis occupies a very extensive range from Alaska to central California. From the distribution records (Menzies, 1954a), it appears that the species is broken up into fresh-water, estuarine and intertidal bay populations throughout its range. Barring the unlikely occurrence of multiple local evolutions, it is probable that the same mechanism(s) has created the *G. o. lutea* and *G. o. oregonensis* "subspecies" over the entire range. Therefore, at least three possibilities concerning the origin of the various forms present themselves. (1) The bay and estuarine-fresh-water forms separated long ago (in the sense of geological time) and both are still capable of distributing themselves across open ocean barriers. (2) The bay form has given rise to the estuarine-fresh-water form recently (or *vice versa*). (3) The morphological, physiological, and ecological differences between the two "subspecies" are salinity-induced and the two forms represent ecotypes of the same species. If the first alternative is true, the two "subspecies" are probably separate species. If the second alternative is true, they may be true subspecies.

There are only two apparent morphological differences between *Gnorimosphaeroma oregonensis oregonensis* and *G. o. lutea*—a difference in the morphology of the pleotelson (Fig. 1) and a difference in body proportions. According to Menzies (1954a), the third pleonite in *G. o. lutea* does not reach the lateral border of the pleotelson, but in *G. o. oregonensis* it does. Examination by the writer of several hundred specimens of each form taken from several localities and various salinities confirms Menzies' observation and shows also that the difference in pleotelson structure is remarkably consistent. Further, there were no intergrades with respect to this diagnostic character. The writer observed that the body in *G. o. oregonensis* seemed relatively broader than in *G. o. lutea.* Measurements of body length and width of specimens of the two "subspecies" from various localities and salinities bore out this observation and showed that there is a significant separation between the two forms in the average length/width ratio of the body.

The osmoregulatory abilities of the two supposed subspecies show striking differences. *Gnorimosphaeroma oregonensis oregonensis* was unable to survive in fresh water, at least under experimental conditions (see Riegel, 1959). *G. o. lutea* lived for several days in all salinities under the experimental conditions.

The ecological differences between the two "subspecies" are well-marked and possibly associated, at least in part, with the above-mentioned physiological difference. *Gnorimosphaeroma oregonensis oregonensis* lives intertidally in bays. During periods of low tide, it remains down in the coarse rubble on the beach, where it is out of water, but in very moist conditions. During high tide, it leaves its hiding places and presumably forages for food. *G. o. lutea* lives in estuaries and fresh-water streams and ponds. It is usually associated with mud and vegetation, but occasionally it may be collected from rocky bottoms of small streams. It lives among the roots of aquatic plants and in cracks in mud, rock, wood, and in burrows made by other animals. Of special interest in regard to the ecological separation of the two forms is the fact that *G. o. lutea* is fully capable of occupying the *G. o. oregonensis* habitat (taking into consideration only the former form's salinity

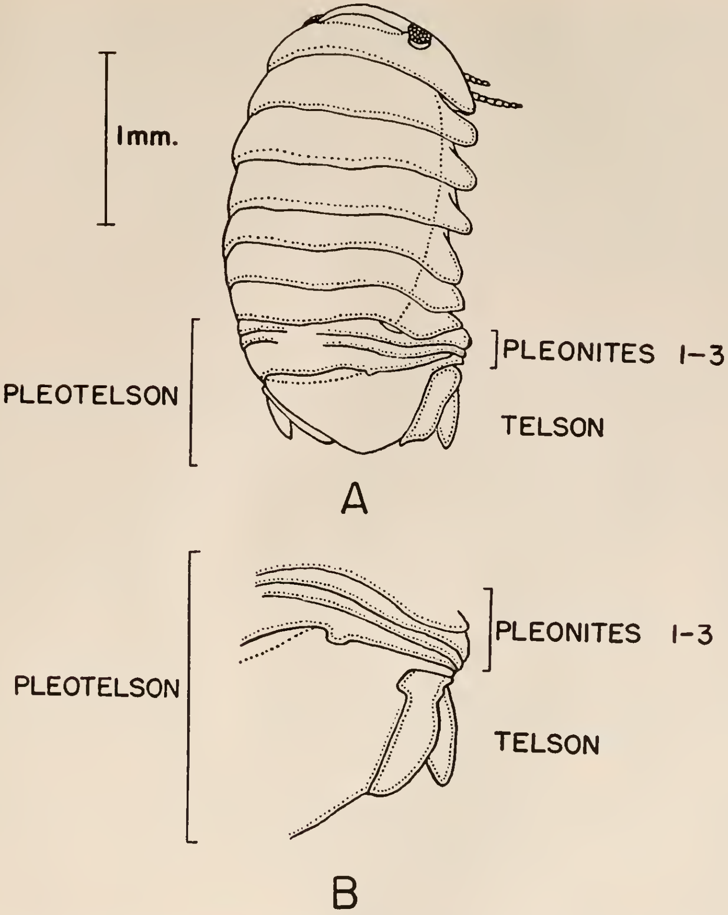

FIGURE 1. A. Diagram of *Gnorimosphaeroma oregonensis lutea* showing the whole animal and the details of its pleotelson morphology. B. Diagram of the pleotelson morphology of *Gnorimosphaeroma oregonensis oregonensis* (A and B after Menzies, 1954a).

tolerance), but it does not do so. Further, as far as can be ascertained, there are no natural hybrids between the two forms.

The osmoregulatory and ecological characteristics of *Gnorimosphaeroma oregonensis oregonensis* and *G. o. lutea* have been considered as adaptive in the sense of Allen (quoted by Pantin, 1932), who suggested that adaptation be measured by survival. From the teleological vantage point afforded by the experiments and observations presented in this paper, it appears that the ability of the two "subspecies" to survive within their respective habitats is correlated with their ability to osmoregulate and survive in various experimental dilutions and concentrations of sea water. Caution must be exercised in making statements concerning the actual barrier(s) or "selective factor(s)" which has permitted one form of *G. orego-*

nensis to invade and exploit a new environment (estuaries and fresh water) to the exclusion of the other. From the experimental data, salinity appears to be a major factor, but it is possible that such is not the case. Prosser (1957) has pointed out that gradual acclimatization to various environmental stresses can often alter an organism's response to those stresses. Anderson and Prosser (1953) showed that specimens of the blue crab, *Callinectes sapidus,* collected from dilute salinities survived better and maintained higher blood osmo-concentrations in dilute sea water than blue crabs collected from higher salinities. However, after acclimatization to 100 per cent sea water for one week, the crabs from dilute sea water approached the crabs from higher salinities in their survival ability and tolerance to osmotic stress. Anderson and Prosser did not indicate whether gradual acclimatization of specimens of *C. sapidus* from high salinities to low salinities would have altered their osmotic behavior. They stated that the observed differences in osmoregulatory and survival ability between the specimens from dilute and more concentrated sea water were due to phenotypic (non-genetic) osmotic adaptation. Lockwood and Croghan (1957) found that fresh-water specimens of the isopod, *Mesidotea entomon,* could be acclimatized to 100 per cent sea water, but specimens of the species taken from brackish water could not be acclimatized to fresh water. Therefore, they felt that the presence of a distinct fresh-water race was indicated. A species of sand worm, *Nereis diversicolor,* has been considered as being composed of physiologically distinct races because of differences in ability to tolerate dilute salinities between specimens from populations over different parts of its wide range. Smith (1955) concluded from studies of chloride ion regulation of specimens of the species from England, Scotland, and Finland that the experimental evidence suggested only non-genetic variation between the specimens from different populations. In the present work, an attempt has been made to determine the effect of salinity on the morphology of the experimental animals when raised under abnormal salinity conditions, but nothing has been learned about the effect of rearing in foreign salinities on the osmoregulatory response of the animals. Adult specimens of *Gnorimosphaeroma oregonensis oregonensis* were gradually (over ten days) acclimatized to low salinities, but they could survive only for less than two days in ten per cent sea water or less.

Referring to the three stated alternatives—are *Gnorimosphaeroma oregonensis oregonensis* and *G. o. lutea* subspecies, ecotypes, or separate species?—the writer presents the following conclusions. The lack of morphological and ecological intergrades between the two forms and their apparently discontinuous distribution rule out, in the writer's opinion, the possibility that they are true subspecies. Since there is no evidence, thus far encountered, to indicate that *G. o. oregonensis* and *G. o. lutea* are ecotypes of the same species, only a remote possibility persists that such is the case. The morphological differences between them are not correlated with any known factor in their environment, which is perhaps not to be expected, but some adaptive relationship is commonly seen in ecotypes. Further, specimens of *G. o. lutea* hatched and reared in habitat conditions close to those of *G. o. oregonensis* retained (at least for three months) their typical morphological configuration. Therefore, the writer is of the opinion that the two "subspecies" are actually species. To establish beyond all question their status as true species, it would be necessary to extend salinity tolerance tests on early developmental stages and immature individuals and to perform breeding experiments on the two forms to establish whether or not actual interbreeding is possible, and if so, whether the hybrids are fertile. Until such time as the above-described tests can be accomplished, the best disposition of the case seems to be to propose that *G. o. oregonensis* and *G. o. lutea* be considered full species. As stated by Prosser (1957, p. 363), "most functional variation among animal populations appears to be either non-genetic or specific; relatively little is racial." Thus *G. o. oregonensis* becomes *G. oregonensis* (Dana, 1852) and *G. o. lutea* becomes *G. lutea* Menzies 1954. For complete descriptions of the two species, a review of their taxonomy and distribution and the disposition of types, the reader is referred to the paper by Menzies (1954a).

The writer wishes to express his gratitude to Professor Milton A. Miller, of the University of California, Davis, for his many comments and suggestions in the period during which this study was in progress. For their generous comments and criticisms during one or another stage in the development of the manuscript, the writer conveys sincere thanks to Dr. Ralph I. Smith, of the University of California, Berkeley, Professor C. Ladd Prosser, of the University of Illinois, Professor Maurice James, of the State College of Washington, and Dr. Robert J. Menzies,[2] of the Lamont Geological Laboratories, Columbia University.

SUMMARY

1. In the writer's opinion, Menzies' determination that *Gnorimosphaeroma oregonensis* consists of two subspecies is not valid, since there is no apparent morphological and ecological intergradation between the two, and their distribution is discontinuous.

2. There is no evidence that the two forms are ecotypes. The morphological differences between them are not correlated with any known factor in their environment, and *Gnorimosphaeroma oregonensis lutea* hatched and reared for three months in habitat conditions close to those of *G. o. oregonensis* retained their typical morphological configuration.

3. It is the opinion of the writer that until such time as extensive rearing and breeding tests can be performed, it is best to propose the elevation of the two subspecies to full species status.

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[2] It seems only fair to state that Dr. Menzies is not in agreement with the conclusions drawn in this paper concerning the elevation of *Gnorimosphaeroma oregonensis oregonensis* and *G. o. lutea* to species status.

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STUDIES ON THE PHYSIOLOGICAL VARIATION BETWEEN TROPICAL AND TEMPERATE ZONE FIDDLER CRABS OF THE GENUS UCA. II. OXYGEN CONSUMPTION OF WHOLE ORGANISMS[1]

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The year 1936 marked a new era in investigating compensatory adaptation of the rate of metabolism in organisms from different latitudes. In this year Sparck, Thorson, and Fox independently reported on intra- and interspecific differences in rate functions of organisms in Greenland, the North Sea, and the Mediterranean Sea. Since then a number of other papers dealing with this subject have appeared and recently Prosser (1955) and Bullock (1955) reviewed this area of study.

Studies of the physiological variation of latitudinally isolated populations of fiddler crabs, genus *Uca,* were undertaken to determine the extent of this variation and to correlate these results with their distribution. The first paper in this series (Vernberg and Tashian, 1959) dealt with a study of the thermal death limits of tropical and temperate zone animals as affected by thermal acclimation. The present paper reports on their rate of oxygen consumption as influenced by starvation, size, season and temperature. Although it may be a question as to what constitutes a valid measure of climatic adaptation, it was felt that the rate of oxygen uptake would reflect changes in the physiological response of a total organism more nearly than studies involving its component parts. Subsequent studies will deal with differences in tissue and enzyme activity in respect to climatic adaptation.

Rather than attempt to review all the literature relating to this general problem, the present paper will be restricted to those papers pertaining to oxygen consumption.

Thorson (1936), comparing metabolic rates of lamellibranchs from Greenland and the Mediterranean, reported the following general facts (p. 121): "Hence it would seem that species with a northerly distribution have a higher metabolism than southerly distributed species of the same genus at the same temperature. . . ." He also found a close correlation between oxygen consumption and habits of animals, in that epifaunal forms have a comparatively higher metabolic rate than digging species and level-bottom species. In addition, he noted that certain arctic

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[2] A portion of this work was done while serving as a John Simon Guggenheim Memorial Fellow.

species are very sensitive to slight increases in temperature. Also working with lamellibranchs, Sparck (1936) obtained similar results.

The work of Fox and subsequent papers in collaboration with Wingfield cite further evidence relating to this problem. When comparing metabolic rates of pairs of marine organisms from Kristineberg, Sweden and Plymouth, England, Fox (1936) found the reverse metabolic-temperature response reported by Thorson and Sparck, *i.e.,* the rates of the warmer-water species were higher than their Swedish counterparts when measured at the normal temperature at which the species were collected. However, he reported that the rate of respiratory movements of southern forms at 16.5° C. was the same as northern forms at 5.5°. In 1937 Fox and Wingfield, studying two additional species, one from northern waters and the other one from more southern waters, observed that the metabolic-temperature curves obtained for whole animals and isolated muscle tissue were parallel. They concluded that the greater rate of oxygen consumption of warm water animals is due to greater non-locomotory metabolism. However, in later papers by Fox (1939) and Wingfield (1939), they stressed that when taking into consideration such factors as body size and season aquatic poikilotherms from the north usually will exhibit a higher rate of physiological function at a given temperature than their more southern relatives. Later Berg (1953) reviewed these results of Fox and Wingfield and concluded that most of the exceptions cited by them actually showed some degree of acclimation.

In 1953 Scholander *et al.* measured the rate of oxygen consumption at various temperatures of 38 species of tropical and arctic poikilotherms, including fishes, crustaceans, insects and spiders. They concluded that there is considerable, but incomplete, metabolic adaptation in aquatic arctic forms relative to aquatic tropical species, while terrestrial insects revealed slight adaptation if at all. (They were of the opinion that no evidence has been found to show that organisms are adapted to temperate fluctuation by being metabolically insensitive to temperature changes.)

Reporting on metabolic rates of the two sub-species of *Uca pugnax* from Trinidad, B.W.I., Florida, North Carolina and New York, Tashian (1956) found marked differences in the response of species from the tropical and temperate zone. Recently Tashian and Vernberg (1958) have elevated these sub-species to the specific level. Data on oxygen uptake of latitudinally isolated populations of *Uca pugilator* have been reported by Edwards (1950) and Démeusy (1957). Roberts (1957a, 1957b) studied the shore crab, *Pachygrapsus crassipes,* from different localities on the west coast of California and Oregon and reported a difference in the resting metabolism which could be attributed to compensation for local temperatures. Results of the present paper, while further substantiating some of the findings of the above workers, also breach the gap between some apparent differences reported by various investigators.

MATERIAL AND METHODS

Fiddler crabs are an excellent group of animals to study as they are abundant over most of the eastern coastline of the Americas and islands of the Caribbean (Rathbun, 1918). The various species have either temperate zone or tropical zone affinities, and, in addition, there is an area of overlap of some northern and some southern forms along the northeast coast of Florida (Tashian and Vernberg, 1958).

Animals used in this study were collected from the following areas: Beaufort, North Carolina, latitude 35°; Alligator Harbor, Florida, latitude 30°; and Jamaica, West Indies, latitude 18°. Experimental studies on fiddler crabs from North Carolina and Florida were conducted either at the Duke University Marine Laboratory or at Duke University, and tropical species were studied at the University College of the West Indies, Jamaica. The following is a brief description of the range and local distribution of the seven species of *Uca* used in this study.

Uca minax (Le Conte). Ranges from Massachusetts to Texas (Rathbun, 1918). In the region of Beaufort, North Carolina this species is typically found in the section of the *Spartina* marsh which is farthest from the banks of the drainage ditches and immediately preceding the *Salicornia-Distichlis* zone.

Uca pugilator (Bosc). Ranges from Massachusetts to Texas (Rathbun, 1918). Usually this species is associated with the sandy-muddy beaches of either the protected areas of the harbor or along the sandy sections of the salt marshes.

Uca pugnax (Smith). Ranges from Massachusetts to northeast Florida (Tashian and Vernberg, 1958). Locally this species is found on mud flats along with *U. pugilator* or in muddy areas of the *Spartina* marsh.

Uca rapax (Smith). Ranges from northeast Florida to Brazil (Tashian and Vernberg, 1958; de Oliveira, 1939). In Florida this species may be found alongside *U. pugnax* or more generally nearer the high tide level in sandy soil. In Jamaica this species was collected in many habitats ranging from sandy soils to mangrove swamps.

Uca mordax (Smith). Ranges from the Bahamas and the Gulf of Mexico to Brazil (Rathbun, 1918). In Jamaica this species was frequently collected on sandy-clay flats and among mangrove roots.

Uca thayeri (Rathbun). Ranges from northeast Florida to Brazil. This species was abundant in muddy banks of drainage ditches where *U. rapax* were frequently caught.

Uca leptodactyla (Rathbun). Found from the west coast of Florida and the Bahamas to Brazil (Rathbun, 1918). A small-sized species which was found only on protected sandy-mud beaches.

The three species of fiddler crabs studied from North Carolina were *Uca minax*, *Uca pugnax* and *Uca pugilator*. In Jamaica, determinations were made on *U. leptodactyla, U. rapax, U. mordax* and *U. thayeri*. *U. rapax* and *U. mordax* came from the Port Henderson area, while *U. leptodactyla* were collected from the swamp near the Morant Point Lighthouse, and *U. thayeri* from Port Morant. Only *U. rapax* were studied from Alligator Harbor, Florida.

After collecting the animals and bringing them to the laboratory, they were rinsed in sea water and placed in aquaria containing about one-half inch of sea water. Animals kept as a general supply were exposed to hamburger, fish and Pablum once or twice a week for about 12 hours and then the sea water was changed. Most of the animals appeared to be feeding and in a good state of nutrition as fecal pellets were readily observed and mortality was low. Individuals to be used experimentally were isolated in marked glass containers and then subjected to the condition of the experiment.

Oxygen consumption was determined by means of standard manometric techniques (Umbreit, 1957). A large-sized respirometer flask (volume about 125 cc.) was connected to a conventional Warburg manometer for all determinations, except in the series of experiments involving the smallest sized species, *U. leptodactyla,* where a flask with a volume of 8 cc. proved to be better. A measured amount of filtered sea water was introduced into a flask containing an organism. The salinity was not measured each time but sporadic measurements gave values ranging from 31 to 35 0/00. In all cases a determination of the rate of oxygen consumption involved only one organism per flask. Ten per cent KOH was used to absorb CO_2. Flasks were not shaken as this understandably proved to be too excitatory to the animals. All results are expressed as μl. oxygen consumed/minute/gram of wet weight. Determinations of oxygen consumption were made over a graded temperature series by the use of a thermally controlled water bath. Preliminary studies showed that the time interval required for thermal equilibration and for the rate of oxygen uptake to reach a somewhat steady level varied inversely with temperature. Only oxygen consumption data which were relatively stable over a period of time were used. The duration of the experiment also varied with temperature: at high temperatures rates were determined over a two-hour period, while at low temperatures eight hours of observation were used.

Recent workers have reported on cycles of oxygen consumption in fiddler crabs which were correlated with time of day, tide, seasons, and other factors (Edwards, 1950, and Brown *et al.,* 1954). In the present study an attempt was made to minimize variation due to rhythmic daily fluctuations by making an equal number of determinations in the afternoon and the morning. It is interesting to note that a preliminary study comparing determinations made on the same animals run in the morning and afternoon did not show any consistent variation. Although no attempt was made to correct for possible tidal influence on metabolism, seasonal variation was observed in some species and this will be discussed later.

The recent thermal history of animals from North Carolina and Jamaica was the same in that their habitat and laboratory temperatures were alike, although the determinations were made at different times of the year. The mean water temperature at Jamaica varies slightly throughout the year, ranging from 80°–82° F. while the range during June–August in the Beaufort area was from 77°–82° F. Work at Beaufort extended from June to September, while studies in Jamaica began in October, 1957, and ended in April, 1958.

In all of these studies only male crabs in the intermolt stage were measured. The criteria of Drach (1939) and Guyselman (1953) were used to determine the stage of the molting cycle.

When oxygen consumption data were plotted on logarithmic co-ordinates with rate of oxygen uptake (weight-specific) against the weight of the organism, a regression equation was obtained of the type

$$\frac{O_2}{W} = aW^{(b-1)},$$

where O_2 is oxygen consumption/unit time, W the body weight (wet weight), and a and b are constants, indicating the intercept and the slope of the regression line in the log-log plot. Additional statistics calculated were the standard error, $S(\log y. \log x)$, and coefficient of correlation (r).

EXPERIMENTS AND RESULTS

Influence of starvation on metabolism

One variable in comparing metabolic rates of animals is the degree of starvation. To determine variation due to this factor, 25 specimens of *U. pugnax* from North Carolina were collected, isolated individually, and maintained at room temperature. First, their rate oxygen consumption was determined after being exposed to food and subsequently determined after the first, third, fifth, seventh, ninth, sixteenth and twenty-first day of starvation. Of the original 25 animals, 21 survived for the entire period while four animals died after the sixteenth day. Results represented in Figure 1 are averages of the 21 animals surviving the entire 21 days of starvation.

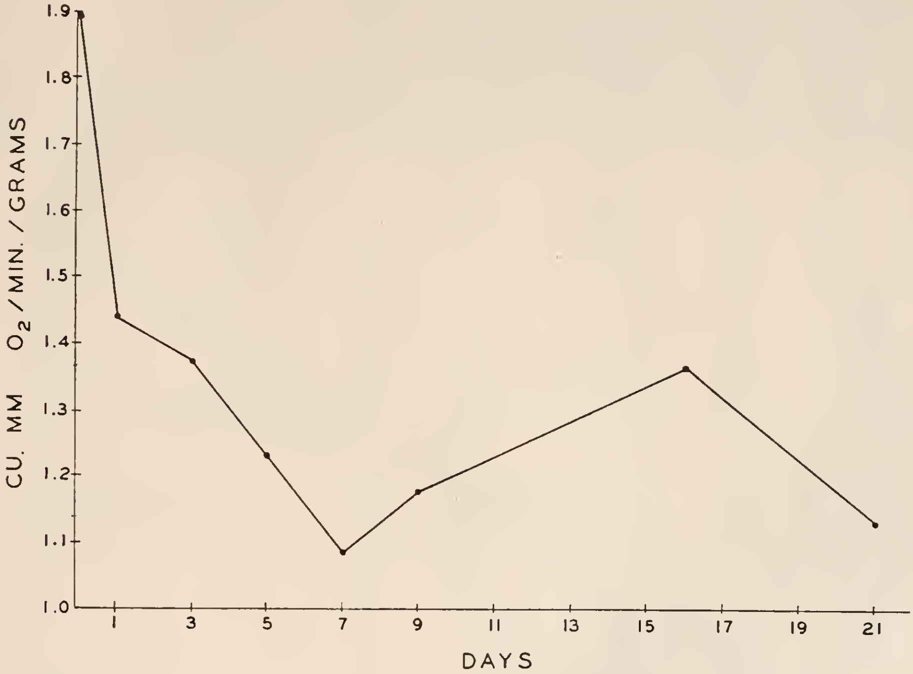

FIGURE 1. The influence of starvation on the rate of oxygen consumption of *Uca pugnax*. Determinations made at 28° C.

A marked drop occurred by day 1 followed by a progressive decline in metabolic rate and subsequent insignificant fluctuations. On the basis of these findings, animals were starved for one to three days before being used in any experiment unless otherwise specified.

The response pattern to starvation of *U. pugnax* is similar to results observed in *Pachygrapsus crassipes* (Roberts, 1957a), pulmonate snails (von Brand, Nolan and Mann, 1948) and fish (Wells, 1935).

Metabolic rates of tropical and temperate zone Uca

Table I represents the rates of oxygen consumption of seven species of fiddler crabs from the tropical and temperate zones determined at different temperatures. Although an increase in temperature generally resulted in a higher rate of oxygen uptake, there appear to be certain thermal ranges within which the metabolic rate is little influenced. A specific example can be seen for *U. pugnax* in that the rate of oxygen consumption at 7° and 12° is similar, but a sharp increase followed when determined at 17°. Throughout the temperature range of 12°–17° (determinations made at 12°, 15°, and 17°), *U. pugilator* and *U. minax* consumed oxygen at about the same rate (Fig. 2). This phenomenon was observed at higher temperature ranges as well for these temperate zone animals; for example, a five-degree increase from 28°–33° only slightly increased the metabolic rate of *U. minax*. This apparent "staircase" effect was noted for tropical animals also, but only at the intermediate and higher temperature levels (Fig. 3).

Changes in the rate of oxygen consumption are expressed as Q_{10}, according to Van't Hoff's equation, rather than the heat of activation (u) of Arrhenius. Q_{10} values for these seven species found in Table II further help to illustrate this "staircase" phenomenon. If determinations had been made only at larger thermal

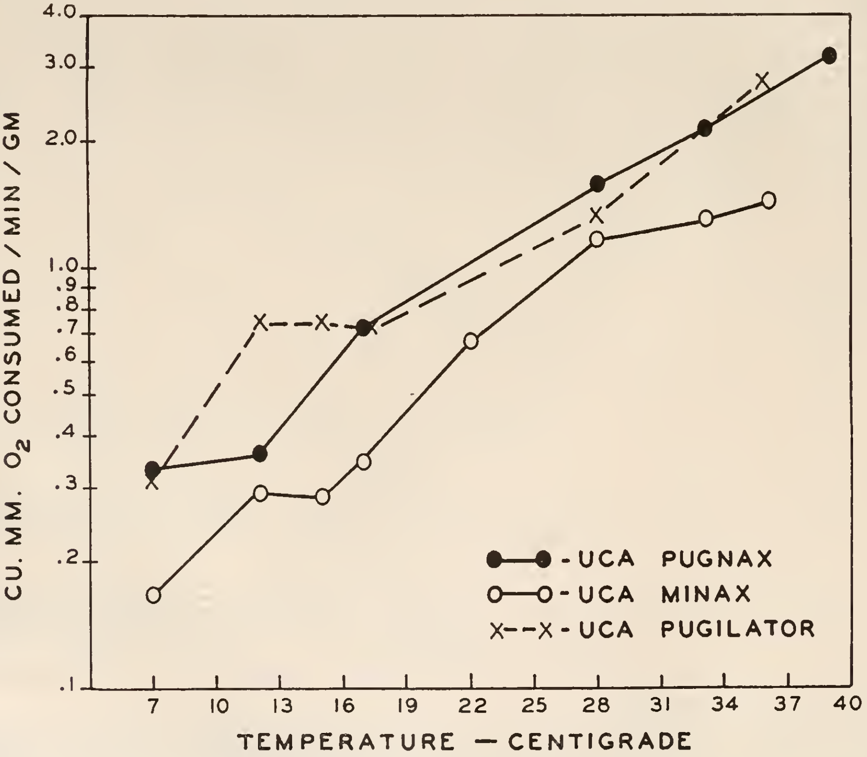

FIGURE 2. The influence of temperature on the rate of oxygen consumption of three species of *Uca* from North Carolina.

TABLE I

Rate of oxygen consumption of Uca from the tropical and temperate zones determined at various temperatures

Species	Temperature (° C.)	Size of sample	Body weight (gms.)		Rate of O_2 consumption (mm.^{3}/min./gram)	
			Mean	Range	Mean	Range
pugnax	7	19	2.57	0.94– 4.72	0.328	0.156–0.725
	12	33	2.70	1.06– 4.65	0.357	0.179–0.730
	17	31	3.63	0.88– 5.95	0.734	0.296–1.410
	28	54	2.90	1.15– 5.57	1.587	0.485–3.489
	33	19	2.95	0.65– 4.31	2.140	1.131–3.705
	39	18	3.20	0.89– 5.57	3.278	2.020–4.523
minax	7	17	6.19	3.85– 8.56	0.167	0.109–0.280
	12	25	6.57	4.54– 9.05	0.292	0.135–0.622
	15	26	6.48	4.79– 8.94	0.281	0.129–0.567
	17	26	6.44	4.58– 8.61	0.342	0.133–0.689
	22	24	6.33	4.41– 8.83	0.688	0.350–1.207
	28	66	5.77	1.66– 8.42	1.185	0.566–2.554
	33	23	6.61	5.04– 8.28	1.308	0.841–1.751
	36	22	6.60	5.01– 8.06	1.442	0.915–1.907
pugilator	7	17	2.06	0.89– 2.84	0.311	0.122–0.627
	12	13	2.03	0.62– 2.95	0.751	0.412–1.482
	15	22	1.87	0.68– 2.95	0.757	0.379–1.395
	17	23	1.93	0.66– 3.41	0.723	0.426–1.264
	28	63	2.35	0.95– 4.87	1.333	0.806–2.473
	36	17	2.06	0.93– 3.00	2.729	1.962–3.896
rapax from Florida	7	24	2.80	1.42– 5.12	0.352	0.150–0.553
	12	32	2.60	1.25– 5.12	0.732	0.280–1.561
	17	30	2.51	1.25– 5.12	1.017	0.396–2.048
	27	97	1.82	0.95– 5.12	2.109	1.125–3.802
	33	13	3.17	1.66– 5.12	2.324	1.558–3.332
	36	25	2.00	1.00– 5.12	2.838	1.315–6.042
rapax from Jamaica	12	25	3.78	0.57– 9.33	0.349	0.167–0.525
	15	40	4.36	0.63–11.57	0.665	0.272–1.877
	17	29	3.51	0.67– 9.33	0.958	0.327–1.931
	22	24	3.10	0.84– 6.56	1.468	0.746–2.369
	28	54	4.33	0.58–13.67	1.663	0.641–4.139
	33	26	3.69	0.67– 9.54	1.954	0.890–3.304
	36	26	3.36	0.58–13.67	2.400	0.801–4.597
	39	23	4.65	0.92–13.67	2.807	0.683–4.497
thayeri	12	11	4.38	1.50– 6.51	0.240	0.149–0.333
	15	12	5.25	2.71– 7.96	0.528	0.383–0.859
	30	13	4.36	2.71– 6.51	1.412	0.675–1.980
	36	16	4.60	1.65– 8.74	1.838	1.306–2.471
mordax	12	9	2.81	1.53– 3.84	0.392	0.216–0.548
	15	7	2.41	0.91– 3.63	0.582	0.462–0.713
	22	8	2.50	0.91– 4.26	1.436	0.884–2.023
	28	12	2.67	0.87– 4.28	1.603	0.942–2.835
	36	11	2.49	0.87– 4.24	2.885	1.457–4.460
leptodactyla	12	11	0.26	0.15 0.31	0.294	0.115–0.545
	15	13	0.28	0.18– 0.35	0.674	0.460–1.176
	30	14	0.27	0.18– 0.36	2.495	1.748–3.768
	36	13	0.28	0.19– 0.35	3.842	2.588–4.973

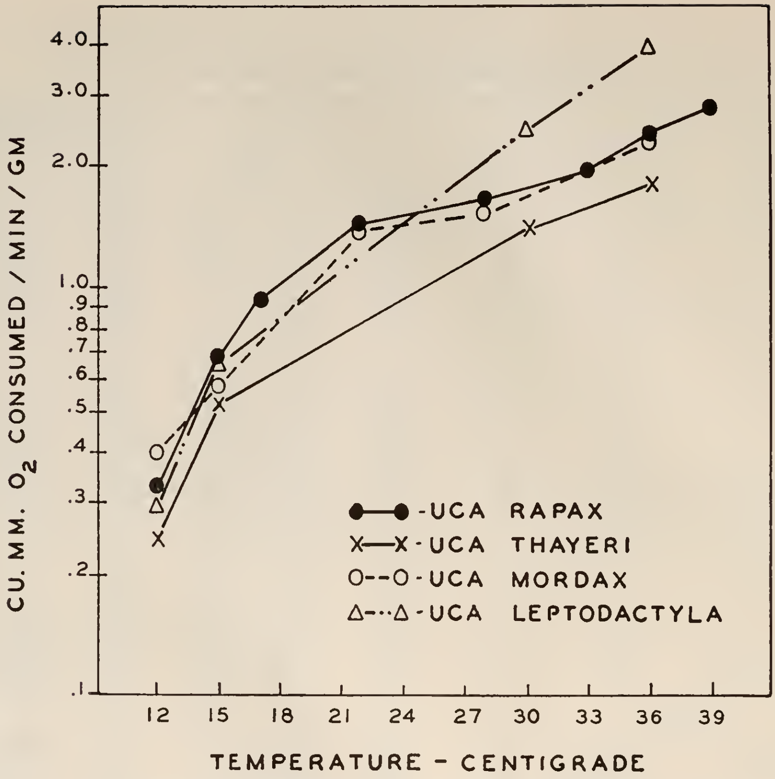

FIGURE 3. The influence of temperature on the rate of oxygen consumption of four species of *Uca* from Jamaica, The West Indies.

intervals, for example every 10 degrees, this type of response would not have been very evident. The Q_{10} value for *U. pugilator* for the temperature range of 7°–17° was 2.32 while a value of 5.83 was obtained for the range of 7°–12°. Tropical species afford additional examples; *U. rapax* had an exceptionally high Q_{10} value of 8.58 over a three-degree increase from 12°–15°, whereas a Q_{10} value of 4.20 for the 10-degree range of 12°–22° was observed.

In general lower Q_{10} values were obtained at higher temperatures than at lower temperatures for all seven species of *Uca* examined. A marked difference in metabolic behavior between tropical and temperate zone animals was observed at the lower temperatures. Between 12° and 15° very high Q_{10} values were obtained for *U. rapax, U. leptodactyla* and *U. thayeri* (range from 8.58–15.9) while a moderately high value of 3.73 was observed for *U. mordax*. Although *U. minax* and *U. pugilator,* both temperate zone forms, showed no increase in metabolic rate within this same three-degree range, moderately high Q_{10} values were obtained between the 7° to 12° range. Determinations made at lower temperatures would probably result in still higher Q_{10} values for these two forms and also for *U. pugnax*. Thus it would seem that at certain points along a temperature gradient, a slight

TABLE II

Q_{10} of oxygen consumption of seven species of Uca from tropical and temperate zones

Temperature range (° C.)	*minax*	*pugnax*	*pugilator*	*rapax* (Florida)	*rapax* (Jamaica)	*mordax*	*lepto-dactyla*	*thayeri*
7–12	3.06	1.20	5.83	4.36	—	—	—	—
7–17	2.04	2.24	2.32	2.89	—	—	—	—
7–28*	2.54	2.10	2.00	2.45	—	—	—	—
7–36	2.10	—	2.11	2.07	—	—	—	—
12–15	1.00	—	1.00	—	8.58	3.73	15.9	13.7
12–17	1.37	4.23	1.00	1.93	—	—	—	—
12–22	—	—	—	—	4.20	3.66	—	—
12–28*	2.40	2.51	1.43	2.02	2.65	2.41	3.28	2.67
12–36	1.95	—	1.71	1.76	2.23	2.27	2.92	2.33
15–17	2.67	—	1.00	—	6.20	—	—	—
15–22	—	—	—	—	3.10	3.49	—	—
15–28*	2.51	—	1.55	—	2.02	2.18	2.39	1.92
15–36	2.18	—	1.84	—	1.84	2.12	2.29	1.68
17–22	4.04	—	—	—	2.35	—	—	—
17–28*	3.09	1.97	1.75	1.86	1.63	—	—	—
17–36	2.45	—	2.01	1.71	1.62	—	—	—
22–28*	2.57	—	—	—	1.23	1.32	—	—
22–33	1.90	—	—	—	1.29	—	—	—
28–33*	1.22	1.90	—	1.42	1.38	—	—	—
28–36*	1.28	—	2.46	1.33	1.57	2.03	2.05	1.55
33–36	2.49	—	—	2.22	1.98	—	—	—

* Determinations were made at 27° for *rapax* (Florida) and 30° for *leptodactyla* and *thayeri.*

increase in temperature results in a marked increase in metabolic rate, whereas at other points the rate of oxygen consumption of these animals is relatively temperature-independent throughout a wider temperature range. Both temperate and tropical species exhibit this type of metabolic response but at different points on the temperature spectrum: tropical animals were metabolically activated at higher temperatures than temperate zone forms.

The similar Q_{10} values for both temperate and tropical zone animals obtained at intermediate and higher temperatures are not surprising as the recent thermal history of all seven species was very similar and reflected summer or elevated temperature conditions. However, at lower temperatures, tropical animals exhibit a reduced ability to meet metabolically this environmental stress, while temperate zone forms appear to be more labile.

When comparing the metabolism of tropical and temperate zone fiddler crabs over a wide range of temperatures, some rather interesting points can be noted. At 12° the range of the average rates of oxygen consumption of the four tropical species is from 0.240 to 0.392 while the range for temperate zone forms is 0.292 to 0.751. These figures would suggest that at this particular low temperature northern species tend to have higher metabolic rates. However, as will be discussed in more detail, it is necessary to take into consideration the factor of body size when comparing species. The following comparisons are of similar sized species: *pugnax-rapax-mordax* and *minax-thayeri.* At 12° *U. pugnax* from North Carolina and *U. rapax* and *U. mordax* from Jamaica consume oxygen at a similar

Table III

Statistical analysis of relation of oxygen consumption to body size of Uca pugnax and Uca rapax determined at various temperatures

Temperature (° C.)	No. of determinations	b-1	log a	$S_{\log y \cdot \log x}$	r
Uca rapax from Jamaica					
12	25	−0.377	−0.301	0.113	0.81
15	40	−0.236	−0.076	0.143	0.42
17	29	−0.571	0.192	0.139	0.75
22	24	−0.387	0.309	0.075	0.82
28	54	−0.343	0.360	0.146	0.64
33	26	−0.377	0.434	0.136	0.67
36	26	−0.374	0.498	0.135	0.77
39	23	−0.354	0.620	0.143	0.69
Uca rapax from Florida					
7	24	−0.050	−0.446	0.133	0.00
12	32	−0.474	−0.010	0.209	0.38
17	30	−0.465	0.150	0.161	0.29
27	97	−0.210	0.356	0.119	0.25
33	13	−0.456	0.574	0.076	0.65
36	25	−0.380	0.557	0.185	0.52
Uca pugnax from North Carolina					
7	19	−0.770	−0.234	0.094	0.88
12	33	−0.537	−0.266	0.102	0.63
17	31	−0.309	−0.003	0.162	0.36
28	54	−0.373	0.321	0.172	0.37
33	19	−0.304	0.423	0.158	0.44
39	18	−0.316	0.650	0.069	0.52

rate. while *U. pugilator* from North Carolina, with a weight average slightly less than these three species, consumed almost twice as much oxygen per unit weight and time. *U. minax,* another northern species, has a higher metabolic rate than *U. thayeri.* Oddly, *U. leptodactyla,* a very small tropical species, consumed oxygen at a rate similar to the larger sized species, while at elevated temperatures (30° and 36°) it had the highest metabolic rate of all seven species.

Determinations were not made at 7° on tropical species as it has been shown by Vernberg and Tashian (1959) that these animals are not able to withstand this low temperature for a long enough period: 50% mortality occurred after exposure to 7° for 30–40 minutes.

As noted above the oxygen consumption rate of tropical species was greatly increased by a three-degree increase from 12°–15° while temperate zone species are little affected.

At 28° *U. pugnax, U. mordax* and *U. rapax* again have similar metabolic rates, while values for *U. thayeri* are now higher than *U. minax* which is the reverse of

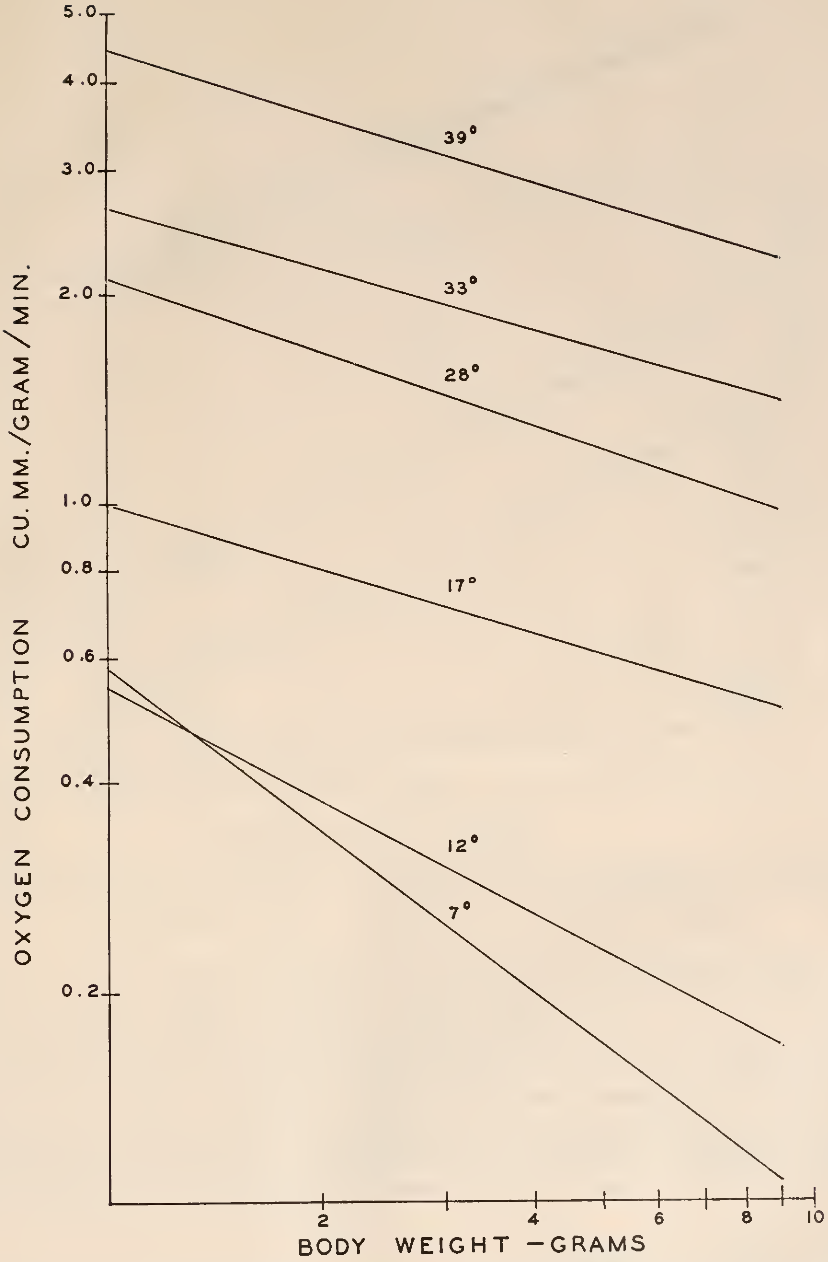

FIGURE 4. The relation of oxygen consumption to size in *Uca pugnax* from North Carolina when determined at different temperatures.

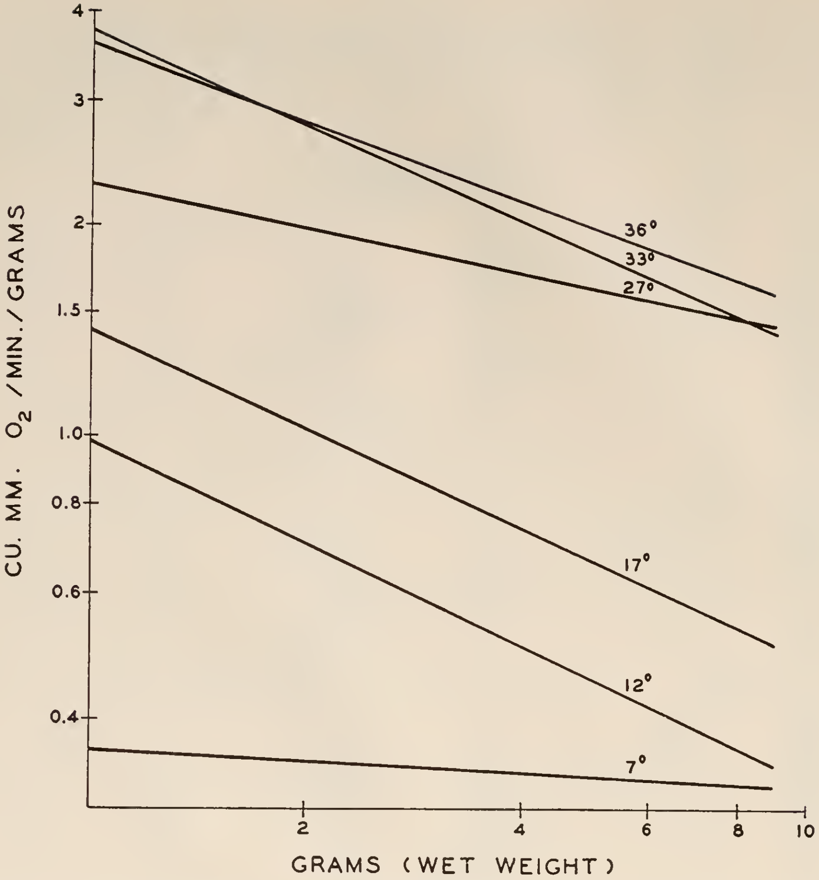

FIGURE 5. The relation of oxygen consumption to size in *Uca rapax* from Alligator Harbor, Florida when determined at different temperatures.

the results obtained at 12°. Although the temperate zone species *U. pugnax* utilized more oxygen than *U. rapax* at 33° and 39°, the tropical species *U. thayeri* consumed oxygen at a faster rate than *U. minax* at elevated temperatures. It would appear that no uniform metabolic difference between tropical and temperate zone species was apparent under the conditions of this study. At any temperature point one tropical species may consume oxygen at a faster rate than a similar sized northern organism, while at this same temperature a temperate zone species of another paired comparison would utilize oxygen faster than its tropical counterpart.

Influence of size on metabolism

Numerous workers have stressed the importance of the dependence of metabolism on body size when making inter- and intraspecific comparisons of crustaceans (Weymouth *et al.*, 1944; Vernberg, 1956; Tashian, 1956; Zeuthen, 1953; Roberts,

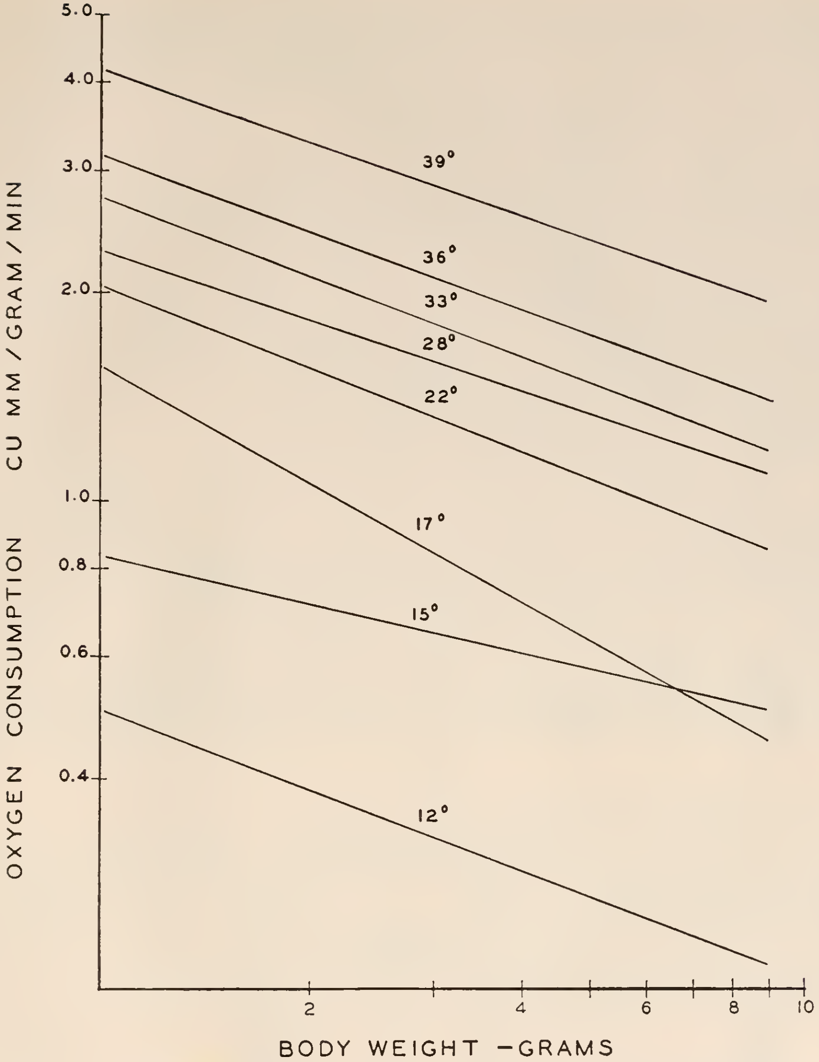

FIGURE 6. The relation of oxygen consumption to size in *Uca rapax* from Jamaica, The West Indies, when determined at different temperatures.

1957a; Edwards and Irving, 1943a, 1943b). In general, smaller individuals or smaller species consume oxygen at a faster rate per unit weight and unit time than larger individuals or larger species.

Oxygen consumption data determined at various temperatures were obtained using different sized animals of *U. pugnax* and two latitudinally separated populations of *U. rapax*. The comparative data for these two species, using the statistical techniques described earlier, are summarized in Table III. Figures 4, 5 and 6 represent the log-log plotting of the regression curves calculated from these data.

It appears that the slope of the regression curve varies with temperature. For *U. rapax* from Jamaica similar *b* values were obtained at all temperatures with the exception of 15°, where the slope was less steep, and 17° where the slope was steeper. The *b* constant for *U. pugnax* also fluctuated with the highest values, being obtained at low temperatures (7° and 12°). Oddly at 7°, *U. rapax* from Florida showed no correlation of body size and respiration, but at 12° the steepest slope was obtained. Although the absolute temperature at which the steepest slope was observed was different for these two species, it appeared that a significant break occurred at some low temperature. Interestingly this break occurred at a lower temperature for the northern species than for the tropical species. In general the slope of the regression curve was less steep for *U. pugnax* than *U. rapax*.

According to the method of Snedecor (1940, pp. 132–133), the correlation coefficients are significant at the 1% level for all points except for *U. pugnax* at 17°, 33°, and 39° where the level of significance is at the 5% level and *U. rapax* (Florida) at 7° where no significance was observed.

When the Q_{10} is calculated from the linear regression curves for animals weighing 1, 3.5 and 9 grams, an apparent difference in response correlated with body size is observed (Table IV). One size group may be more sensitive to a given temperature change than a different size group, *i.e.,* large specimens of *U. rapax* have a larger Q_{10} value than small sized (one gram) individuals at the temperature interval of 12°–15°, while at 15°–17° the reverse is observed.

After a log-log plotting of metabolic data of seven species of fiddler crabs obtained at two temperature levels, interspecific differences correlated with body size are evident (Fig. 7). At 28° or 30° a regression curve with a slope of − 0.204 was obtained which indicates that the small sized species consumes oxygen at a

TABLE IV

Q_{10} values of different sized Uca pugnax and Uca rapax based on linear regression curves

Temperature interval in ° C.	1 gram	3.5 grams	9 grams
Uca pugnax			
7–12	1.0	1.6	2.4
12–17	3.4	6.0	9.2
17–28	2.0	2.9	1.8
28–33	1.6	1.8	2.1
33–39	2.4	2.3	2.2
Uca rapax			
12–15	5.6	12.3	16.1
15–17	24.8	2.2	1.0
17–22	1.9	2.6	2.9
22–28	1.2	1.4	1.0
28–33	1.4	1.3	1.2
33–36	1.6	1.7	1.7
36–39	2.5	2.6	2.9

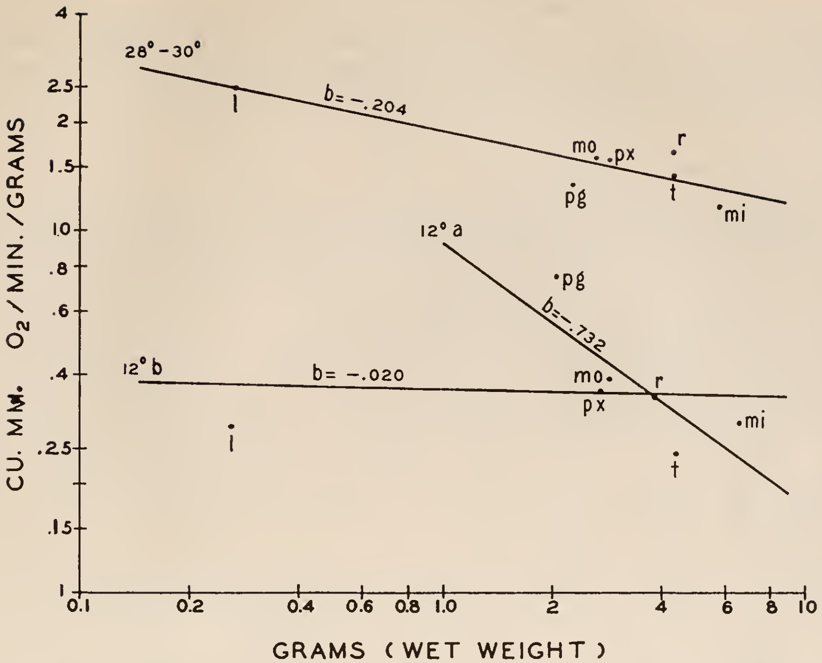

FIGURE 7. The relation of oxygen consumption to size in seven species of *Uca* measured at two temperature levels. Two regression curves were calculated for data at 12°: *a* did not include *leptodactyla* (1) whereas *b* did (see text for discussion). 1 is *leptodactyla*, mi is *minax*, mo is *mordax*, pg is *pugilator*, px is *pugnax*, ra is *rapax*, and t is *thayeri*.

higher rate than a larger sized species. However at 12° the metabolic rate of the small sized tropical species is greatly reduced in relation to the other six species and the regression curve has a slope of − 0.038 which is similar to results obtained for *U. rapax* (Florida) at low temperatures. But, if *U. leptodactyla* data are omitted on the basis of this tremendous response to environmental temperature stress, a curve with a slope of − 0.732 is obtained which seems to correspond to the steep slopes at low temperatures for intraspecific data on *U. pugnax* (a slope of − 0.770 at 7°) and *U. rapax* from Jamaica (a slope of − 0.571 at 17°).

Comparisons of metabolism of different populations of Uca rapax

Data on the oxygen consumption of *U. rapax* from Florida and Jamaica are included in Table I. One of the most obvious differences between the two populations is seen in their respective responses at reduced temperatures. The rate of oxygen uptake of Florida animals at 7° was very similar to that of Jamaican forms at 12°. As noted previously data for Jamaican animals at 7° could not be obtained as they did not survive exposure to this temperature. Following in this same trend, Florida animals consumed oxygen at a slightly higher average rate at 12° than their more tropical counterpart did at 15°. At all subsequent similar temperatures the subtropical form exhibited the highest metabolic rate.

U. rapax from Florida metabolically behaved like *U. pugnax* at the low temperature of 7° while at 12°, 17°, 27° and 33° *rapax* had a higher rate. The Floridan *U. rapax* exhibited the highest Q_{10} value at 7°–12° which is more characteristically like temperate zone forms than tropical zone species.

Influence of season on metabolism

Although results discussed up to this point were made on tropical and temperate zone animals which had similar recent thermal histories, experiments were carried out at different seasons of the year. Therefore the possibility of seasonal fluctuations in metabolism had to be investigated. Although metabolic studies involving Jamaican species were conducted from October to April, it was assumed that if any seasonal variation was to be observed it would be in evidence during this period as there is little monthly variation in temperature throughout the year.

When oxygen consumption determinations on *U. rapax* made either at 28° or 15° during October were compared with results of February–March, there was no significant difference of means. This indicated that under the conditions of these observations no shift in metabolism of *U. rapax* was observed which could be correlated with seasons (Table V).

Data on *U. pugnax* collected during the summer months and also during the period from November to January show a marked seasonal variation. At 7°, 17° and 28°, crabs collected during November and maintained in the laboratory under the same thermal conditions as summer animals (22°–27°) consumed oxygen at a significantly faster rate than summer animals run at the same temperatures. However, winter and summer animals responded similarly at 33°. The Q_{10} of 1.61 of "winter" animals was lower than that of summer animals (2.24) at low temperatures (7°–17°) but the opposite response was observed at higher temperatures (17°–28°) where the respective values were 2.37 and 1.97.

TABLE V

Metabolic rate of Uca pugnax determined at different seasons of the year and at various temperatures

Month of year	Temp. (° C.)	No. of determinations	Oxygen consumption rate (mm.³/gm./min.)	Standard error of mean	Level of significant difference of means
June to August	7	19	0.328	0.035	>.01 highly significant
November to January	7	23	0.572	0.039	
June to August	17	31	0.734	0.047	>.01 highly significant
November to January	17	40	0.920	0.052	
June to August	28	54	1.587	0.094	>.01 highly significant
December to January	28	25	2.379	0.222	
June to August	33	19	2.140	0.207	no significant difference
December to January	33	27	2.312	0.113	

Discussion

Results of this investigation demonstrate the existence of differences in one physiological response, oxygen consumption rate, between temperate and tropical zone fiddler crabs. Other workers, using different measures of climatic adaptation, have reported the existence of physiological variation between latitudinally separated species of poikilotherms (Mayer, 1914, pulsation rate of the bell of *Aurelia aurita;* Hörstadius, 1925, Thorson, 1936, Moore, 1939, 1942, 1949, Dehnel, 1955, egg development and growth of various invertebrates and frogs; and Rao, 1953, rate of ciliary pumping of water in a mussel).

When making intra- or interspecific comparisons of poikilothermic animals from different latitudes, it is generally stated that at any given temperature, within limits a northern or cold-adapted form will show a higher metabolic rate than a southern or warm-adapted form. This comparison is dependent upon a number of other factors, such as body size and season, as pointed out so excellently by Prosser (1955), Bullock (1955) and Rao and Bullock (1954).

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However, when comparing similar sized species of fiddler crabs from temperate and tropic zones, which have similar thermal histories, no consistent difference in metabolism correlated with latitude was observed except at low temperatures. But an intraspecific comparison of *Uca rapax* from northern Florida and Jamaica shows the classical type of response, especially at low and high temperatures. Using the data of Tashian (1956) it can be seen that similar sized fiddler crabs (3 gms.) from southern Florida and Trinidad had similar metabolic rates when determined at 24°, while the New York species was slightly lower. But at 14.1°–14.9° C., animals from New York weighing 3 gms. had a higher metabolic rate than the more southern forms. Working at still lower temperatures (1.4° and 15° C.) Démeusy (1957) reported that *Uca pugilator* from Woods Hole, Massachusetts had a significantly higher rate of metabolism than specimens from Florida only at the lower temperature. It is possible that these results might be influenced by seasonal temperature changes as this work was started in the fall. Whereas Démeusy did not find any difference at 15° between these two populations, Edwards (1950) reported the Woods Hole form of *U. pugilator* to have a higher rate of oxygen consumption at 20° than animals from Florida. No mention was made of either the thermal or seasonal history of the two populations studied. However, seasonal studies on *U. pugnax* indicated that "winter" animals from North Carolina had higher metabolic rates in the temperature range of 7°–25° than "summer" animals, while the tropical species, *U. rapax,* did not show any seasonal fluctuation. This absence of any seasonal variation in metabolism of *U. rapax* may be correlated with the thermal constancy of their environment throughout the year. However, fluctuating yearly temperatures of more northern latitudes have resulted in a labile metabolic pattern in *U. pugnax* which can be correlated with thermal acclimation.

Teal (1959), dealing with the relation of the respiratory metabolism of crabs to flow of energy through an ecosystem in Georgia salt marshes, found a marked ability of *U. pugnax* to demonstrate seasonal thermal acclimation.

The review paper of Bullock (1955) cites numerous examples of seasonal acclimation in many but not all poikilotherms. Recently Roberts (1957b) reported that in the lined shore crab, *Pachygrapsus crassipes,* seasonal acclimation in metabolism was not present when determination was made at 16° C. However, he noted that respiration rates did bear some relationship to local seasonal temperature changes when intertidal sea water temperatures were below the environmental mean of 16°. It is noteworthy that the annual temperature flutuation experienced by *Pachygrapsus* is much less than that of fiddler crabs from North Carolina and thus might explain the difference in degree of response. The high and low mean monthly average temperature reported by Roberts was about 12° in January and 22° in August. At Beaufort McDougall (1943) and Gutsell (1930) reported similar average temperature values at 5.5° in February and 28° in July.

Metabolic rate determinations made at a number of temperature levels give a better insight into the influence of temperature on metabolism than generalizations from a few widely separated thermal points. The results of the present paper show that certain points along a temperature gradient appear to be of a more "critical" nature for the organism than others. This type of metabolic response when graphed gives a "staircase" appearance. In general tropical and temperate fiddler crabs responded similarly at intermediate and elevated temperatures, but at low temperatures tropical species were metabolically activated at a higher temperature than more northern species. Woodworth (1936) and Vernberg and Mariney (1957) observed that terrestrial insects, bees and fruit flies, respectively, were relatively temperature-insensitive within a given temperature range. Takatsuki (1928) showed a similar response in the heart rate at various temperatures of oysters from the tropical and temperate zone seas. When comparing the results of this study with those of Teal (1959), a remarkable similarity is observed. To cite a few examples: Teal observed *U. minax* consumed oxygen at the same rate at 11.1° as at 15.9°; in the present study the temperature range of 12° to 17° had little effect on their metabolism. *U. pugilator* was found to be temperature-insensitive between 13.2° and 19.4° by Teal and in the present investigation the same response was noted from 12° to 17°. Noteworthy is the similarity of the response of *U. pugnax*. In both studies, this species behaved differently than the other two species of *Uca:* while *pugilator* and *minax* were temperature-insensitive in this range of about 11°–19°, the metabolic rate of *pugnax* was greatly increased. Additional cases, chiefly terrestrial animals, are cited by Bullock (1955).

In general this marked influence of a relatively narrow temperature increase may be expressed in terms of a high Q_{10} (values greater than 3). After reviewing and re-evaluating many papers, Rao and Bullock (1954) concluded that Q_{10} was dependent on size and temperature of adaptation. Results of the present paper present additional data to show that marked differences in Q_{10} exist in the semi-terrestrial crabs of the genus *Uca* from the tropical and temperate zones. Scholander *et al.* (1953) measured the metabolic rate of *U. mordax* from Panama and reported high Q_{10}'s at low temperatures. However it is difficult to compare results for the following reasons: 1) determinations were made only at 10-degree intervals; 2) Q_{10}'s were estimated by eye-fitted tangents to eye-fitted curves; and 3) few data were available at 10°, as only three out of eight animals survived sufficiently long to give valid readings.

Similar results as the present paper were obtained by Thorson (1936), the most striking example being a Q_{10} of 21 over the temperature range of –1° to 1° for *Pecten groenlandicus*. Interestingly this lamellibranch lives in the fjords of Greenland where the temperature is constantly below 0°. The same type of response appears in the results of Sparck (1936) but it is difficult to understand the basis of his metabolic-temperature curves as no experimental data are given and no points are shown on the curves. Démeusy (1957) and Teal (1959) observed a similar pattern of Q_{10} values with their work on *Uca*.

Results of the present paper demonstrate the dependence of metabolism on body size both when making inter- and intraspecific comparisons. The slope of the linear regression (b-1) varies with temperature: the steepest slope is at a higher temperature for the tropical species than for its northern counterpart. Although there are only a few observations, these results show the same tendency of temperature to influence metabolism as did Q_{10} values for the 7 species. Roberts (1957a) observed that the slope of the linear regression was the same at 8.5° and 16° for *Pachygrapsus* but was significantly less steep at 23.5°. In their recent review paper, Rao and Bullock (1954) replotted the data of Edwards and Irving (1943a) and Edwards (1946) and found that the slope varied with temperature: 1) with summer animals, the slope was steeper at 12° than at 22°; and 2) with winter animals the reverse was noted. As a consequence of the change in slope of the regression curve, the Q_{10} values of different sized organisms will be changed. Bishop (1950, p. 242) reported that smaller individuals of a species are more temperature-sensitive than larger ones, while Rao and Bullock (1954) concluded that commonly the Q_{10} increases along with increasing size within normal ranges of temperature. Results of the present paper show that the influence of temperature on the metabolism of different sized individuals of one species varied with the temperature range which was used. Therefore apparent differences reported in the literature may have resulted from comparing different temperature ranges of animals.

Interspecific comparisons of fiddler crabs showed the slope of the linear regression to be – 0.204 at 28°–30° which compares favorably with slopes obtained for various groups of organisms (Zeuthen, 1953, – 0.20 for crustaceans; Weymouth *et al.*, 1944, – 0.174 for various crustaceans; and Vernberg and Hunter, 1959, – 0.21 for cercariae of digenetic trematodes). But, when compared with the results at 12° C., marked differences are observed. The metabolic rate of the smallest species was depressed greatly by reduced temperature resulting in a regression slope of – 0.020. Hence, smaller sized fiddler crabs have higher Q_{10}'s than larger sized species with the temperature range of 12° to 28° or 30°.

Results of this paper would suggest that the metabolic response of fiddler crabs has real significance to their distribution. In the course of evolution, the various populations studied appear to be metabolically adjusted to the temperature fluctuations of their habitats. Temperate zone species not only are metabolically active at lower temperatures than tropical species but they exhibit a seasonal cycle as well. These differences are not only very marked when making interspecific comparisons but intraspecific differentiation is observed. The northernmost population of *U. rapax* behaves more like a temperate zone species at low temperatures than its southern relatives.

SUMMARY

1. The rate of oxygen consumption of seven species of *Uca* from the tropical and temperate zones was determined over a graded temperature series. All species had a similar recent thermal history.

2. Starvation resulted in an initial decrease in metabolic rate followed by a relatively constant rate in *Uca pugnax*.

3. Generally increased temperature resulted in increased rates of oxygen consumption. However, in some cases a given temperature range had little or no effect on metabolism, while at other temperatures a marked increase resulted.

4. Q_{10}'s of temperate and tropical zone species were similar at intermediate and higher temperatures but differed at lower thermal levels. Q_{10} varied with size and temperature levels. Lower Q_{10}'s were obtained at higer temperatures.

5. Intra- and interspecific comparisons of metabolism-size relationships were made on data obtained at various temperatures. The slopes of the regression varied with temperature and species.

6. When comparing the metabolic response of two latitudinally isolated populations of *Uca rapax* with a closely related temperate zone species, the pattern of the northernmost population of *U. rapax* was intermediate between the tropical and temperate zone forms.

7. Although no seasonal variation in metabolism was observed in tropical species, fluctuation was observed in a temperate zone species.

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AUTHOR'S ERRATA

In the paper by Giese *et al.*, which appeared in Volume 116, No. 1, of THE BIOLOGICAL BULLETIN, for February, 1958, the following errata occurred:

Page 53, Table II: The first sign under the column heading "Sex and condition" should be "♀ spent," not "♂ spent."

Page 53, Table II: The next to last number under the column heading "Gonad index" should be *17.7*, not *1.77*.

Page 56, footnote 5, line 4: Insert at the beginning of the line the words "the variability in."

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STUDIES ON THE CARDIAC STOMACH OF A STARFISH, PATIRIA MINIATA (BRANDT)[1]

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Patiria miniata, the cushion star or sea-bat of the Pacific Coast of North America, is perhaps best known to zoologists as a ready source of materials for the study of asteroid embryology. Of equal interest, however, are its voracious appetite and its unusual feeding habits. *Patiria* functions as an omnivorous scavenger of both plant and animal materials and to some degree at least as a predator on sessile gastropods. Unlike *Asterias* and *Pisaster,* it does not open bivalves; but it has developed to a much greater extent than any of the free-armed starfishes the ability to evert the cardiac stomach through the mouth and to employ it as a feeding organ of great effectiveness. Almost every individual observed undisturbed in a tide pool is found with its remarkably voluminous cardiac stomach fully everted and widely spread. Specimens maintained in an aquarium spend a good share of the time with their everted stomachs closely applied to the glass, perhaps, as suggested by MacGinitie and MacGinitie (1949, p. 227), digesting accumulated growths of diatoms. Bits of kelp or other seaweed, cracked snails, large pieces of mussel flesh, living limpets, or even smaller specimens of *Patiria* are covered and held against the substratum by the body of the starfish, then enveloped by the spreading folds of the everted stomach (Fig. 4) until completely digested. Even small pieces of soft food, which the animal is capable of ingesting, are first covered and wrapped in folds of the stomach before being brought into the cavity of the gut.

Such observations suggest that the cardiac stomach of *Patiria* functions in a highly specialized manner, being more frequently and more extensively exposed to the external environment than the stomachs of other familiar starfishes. It seemed likely that the functional peculiarities of this organ might be reflected in significant structural specializations, probably involving regional differentiation of the stomach wall and a particularly well-developed system of fibrous attachments for holding and retracting the everted stomach. Although *Patiria* is a relatively common intertidal species in much of its range along the Pacific coast, no detailed anatomical or histological studies of its digestive tract appear to have been made. The present

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[1] These studies form a part of a program of investigation begun at the Hopkins Marine Station of Stanford University, Pacific Grove, California, during the tenure of a John Simon Guggenheim Memorial Fellowship. They were supported by USPHS Grant No. RG-5755 and by funds from NSF Grant No. G6007 to Cornell University.

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study was undertaken to provide a basis for comparison in a contemplated series of experiments, and as a contribution to knowledge of comparative and functional anatomy and histology in the digestive systems of asteroids. The results of this study have brought to light several interesting features of the cardiac stomach of *Patiria* that have apparently not been described previously, either for this species or for any of its relatives.

It is a pleasure to express to the Director and Staff of the Hopkins Marine Station my appreciation for their hospitality and helpful cooperation during the conduct of these investigations.

Material and Methods

Small to moderate-sized specimens of *Patiria miniata* were collected in tide pools near Pacific Grove, California, and maintained in the laboratory in running sea-water. Many observations of normal feeding behavior were made on these animals, which were periodically provided with a variety of food such as cracked snails, opened mussels, kelp fronds, living limpets, etc.

For anatomical studies, animals were immersed in isotonic $MgCl_2$ (8% in tap water) until flaccid, then opened by cutting through the body wall all around the margin of the body and removing the aboral part, transecting the digestive tract at the junction between cardiac and pyloric stomachs. The cardiac stomach and its retractor harness were then studied in their normal positions; or, by cutting all the strands binding the stomach to the ambulacral ossicles and transecting the esophagus, the stomach could be removed from the body. Portions of such excised stomachs, maintained in a relaxed state by continued immersion in $MgCl_2$ solution, were spread and pinned out with fine glass needles in small wax-bottomed dissecting trays. After completion of studies of these spread segments in the living condition, the salt solution was decanted and the preparation flooded with Helly's fluid or Zenker-acetic. Fixation was continued for 24 hours; the fixed tissue was then removed from the wax, washed for several hours in running water, trimmed, dehydrated, and imbedded in paraffin. Carefully oriented serial sections were cut at 7 μ, in a plane parallel to the mouth. These "horizontal" sections were carried through a variety of staining routines, for various purposes. For general histological observations, Harris' hematoxylin followed by eosin or light green was used; for delineation of cell boundaries, cytoplasmic granules, and muscular and connective-tissue fibers, Mallory's phosphotungstic acid hematoxylin (PTA) proved excellent. Glycogen and other polysaccharide complexes were demonstrated by the use of a periodic acid-Schiff routine (Lillie, 1954, p. 123), controlled by salivary digestion. Metachromatic substances were revealed by overnight staining in dilute toluidine blue, followed by alcoholic dehydration and differentiation. A standard Feulgen technic was used to identify nuclei by selective staining of their DNA content.

Certain special experimental procedures will be described later, in connection with the observations they were designed to elucidate.

Observations

The retracted cardiac stomach occupies a large part of the central cavity of the disk. It lies in the form of a more or less regular series of pouches, extending from the main lumen both perradially and interradially, above and between the proximal ambulacral ossicles of the rays. These pouches are separated by medially and upwardly directed folds that extended almost to meet a series of similar folds hanging from the roof of the pyloric stomach. A relatively slight constriction divides the stomach into the conventionally recognized cardiac and pyloric portions, leaving a comparatively broad passageway between them; it is into this passageway that the medial folds of the cardiac stomach protrude. The constriction separating the upper and lower portions of the stomach is girdled by a glistening band of connective-tissue fibers related to the retractor harness of the cardiac stomach.

The retractor mechanism

What may be termed the extrinsic elements of the retractor harness consist in each ray of a pair of fan-shaped bundles extending from broad origins along the sides of the ambulacral ridge to rather more restricted attachments on the wall of the cardiac stomach (Fig. 2). These bundles bear a superficial resemblance to the extrinsic strands described for *Asterias forbesi* by Anderson (1954) but differ markedly in detail. Whereas in *Asterias* the extrinsic components consist of thick marginal bands supporting thinner, mesentery-like sheets, those of *Patiria* are thick and tough throughout, and each of the fan-shaped bands comprises four strands. The shortest and most proximal of these originates on the connective-tissue coat lateral to the first or proximal ambulacral ossicle and follows a short course aborally, skirting between the perradial and interradial pouches, to insert on the fibrous girdle described above. The longest of the extrinsic strands forms the upper border of the fan-shaped band, originating lateral to the ambulacral ridge in the region of the tenth to twelfth ossicle and inserting also on the fibrous girdle of the stomach, adjacent to the attachment of the shortest strand. Between the shortest and the longest lie two intermediate strands, somewhat thicker, originating alongside the ambulacral ridge, passing between the perradial and interradial pouches, and disappearing proximally under the perradial pouch. Here these strands appear to end rather abruptly but actually break up into branching and rebranching intrinsic retractor fibers spreading widely over the surface of the cardiac stomach as they approach the oral end of this organ. It will be noted that the stomach of *Patiria* lacks the nodules characteristic of the retractor system of *Asterias,* the conspicuous fibrous knots upon which all extrinsic strands insert and from which all intrinsic strands branch. Instead, in *Patiria* the stomach is provided with the fibrous girdle which is related only to those elements of the extrinsic system that do not contribute to the branching intrinsic fiber systems.

The intrinsic fiber patterns on the wall of the cardiac stomach in *Patiria* generally resemble those of *Asterias.* In the portion of the stomach pertaining to each of the rays, two major bands pass from the blunt ends of the intermediate extrinsic strands, bound together initially but immediately diverging widely to course diagonally downward. These soon become closely applied to the outside of the stomach and pass underneath the visceral peritoneum to establish contact with the muscular and connective-tissue layers of the stomach wall. Each of the major strands now branches into two, and below the points of divergence of the four strands thus produced a series of four additional dichotomies occurs. Eventually, the final products of all these binary divisions, tapering slowly and growing progressively smaller as they approach the lower end of the stomach, disappear as fine fibers blending into the stomach wall at a line near the upper limit of the esophagus. The paired extrinsic bands in each of the rays are thus continued as intrinsic strands intimately connected with the fabric of the stomach wall and spreading to 64 widely dispersed terminal attachments at its lower end; in a 5-rayed specimen, the intrinsic retractor fibers are distributed to a total of 320 ultimate points of attachment, all arranged along a regular line (*cf.* Fig. 1). For purposes of identification in subsequent discussions and to avoid confusion with other components to be described later, the branching strands spreading from the ends of the extrinsic bands will be referred to as *Class 1* fibers.

Histological examination shows that these intrinsic strands, like those of *Asterias,* are composed of varying mixtures of connective-tissue and muscle fibers, bound together and enclosed by the typical cuboidal epithelium of the visceral peritoneum (Fig. 7). As the strands branch, becoming smaller and more intimately connected with the stomach wall, they continually contribute both connective-tissue and muscle fibers to the corresponding layers in the wall of the stomach (Fig. 8). In their lowest levels, near the esophagus, the small terminal branches can be recognized only in sections, appearing as characteristically staining, somewhat thickened groups of connective-tissue fibers running longitudinally beneath the peritoneum. The larger Class 1 fibers are attached to the stomach wall only along their sides, often leaving a long, tubular cavity beneath the strand. In such spaces, the outer surface of the stomach appears to be composed of its muscular and connective-tissue coats, as the retractor strands run inside the peritoneum (Figs. 5, 6, 17).

The Class 1 fibers, in addition to making direct contributions to the muscular and connective-tissue layers of the stomach, are also closely related to a secondary system of strands to be designated as *Class 2* fibers. These are found in a zone lying roughly between the second and fifth forks of the Class 1 system. They consist of very large numbers of tiny, parallel strands which spring at closely spaced but irregular intervals from the stomach wall, in close proximity to the courses of Class 1 fibers (Fig. 6). The Class 2 strands run a horizontal course, leaving the stomach wall to branch perhaps once before re-entering the wall and disappearing again, in the unsupported pouches between the Class 1 branches (Fig. 3). The horizontal fibers are longest high in the stomach, where they arise near the widely separated major branches of the Class 1 system; they grow progressively shorter in lower levels, where the Class 1 branches approach each other more closely. The small and inconspicuous Class 2 fibers are of particular interest in that they constitute a previously undescribed feature of the retractor mechanism and one that does not exist in *Asterias.*

Study of the Class 2 fibers in histological sections shows that they originate in the muscle layers of the wall of the stomach and hence are formed almost exclusively of strands that can be identified as muscle fibers (Fig. 6). These join and pass out through the more superficial connective-tissue layer to form cylindrical bundles of parallel fibers, each enclosed by a layer of peritoneal cells (Fig. 9). After a longer or shorter course through the perivisceral coelom near the outer surface of the stomach, the strands re-enter the wall, penetrate the connective-tissue layer again, and contribute their fibers in a spreading pattern to the muscle layers of the stomach (Fig. 10). Class 2 bundles in their free courses over the surface of the stomach range approximately from 9 to 14 μ in diameter.

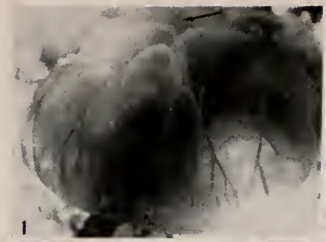
Still another group of fibers is found outside the stomach of *Patiria*, lying in parallel longitudinal array about its lower end. These, to be referred to as *Class 3* fibers, are the most slender of all the retractor and restraining bands of the stomach wall. Each appears to consist of a single, hyaline, homogeneous strand of extracellular fibrous material enclosed by the vastly extended cytoplasm of the cell that produced it; the nucleus of the cell lies in a thickened area of the cytoplasm somewhere along the length of the strand (Fig. 13). Class 3 fibers exist in a considerable size range, from just under 2 μ to more than 8 μ in diameter. The coarsest are also the longest, originating in the connective-tissue components of small Class 1 fibers, passing out through the peritoneum into the coelom, turning downward toward the peristome, and finally penetrating the peritoneum again to contribute several branching processes to the connective-tissue layer of the esophagus or peristome. The origin of such a coarse fiber from a small Class 1 strand is shown in Figure 11; Figure 12 shows the insertion of a similar strand in the peristomial region. The most numerous of the Class 3 fibers are smaller and shorter, having both ends anchored in the connective-tissue layer of the stomach itself, not related to other strands. These run brief courses across folds in the stomach wall (Fig. 13) or across the deep bay formed by the reflection of the esophagus above the horizontal peristomial membrane. It should be emphasized that all of the Class 3 fibers, of whatever size, consist of single, nucleated cells enclosing fibrous extracellular strands; they contain no muscle fibers, and they are never clothed by a peritoneal layer as fibers of Classes 1 and 2 always are. Like the Class 2 fibers, these constitute a new feature, previously undescribed.

In a restricted zone at the lowest end of the stomach, fibers of all three classes are present; that is, Class 1 fibers give off coarse Class 3 fibers in the zone near the esophagus in which short Class 2 strands also occur. Here, of course, the horizontal Class 2 fibers and the longitudinal Class 3 strands run perpendicular to each other. By far the majority of the Class 3 fibers, however, are found below the level occupied by the lowest of the Class 2 strands.

The stomach wall

In *Patiria*, as in *Asterias*, the spreading branches of the intrinsic fibers (Class 1) are accompanied by underlying specializations in the structure of the stomach wall. These consist of branching gutters, beginning at the lower end of the stomach beneath the terminal branches of the Class 1 fibers and running upward, converging and joining with each other to form progressively larger channels that ultimately widen and fade out at approximately the level where the primary strands of the intrinsic retractor system become attached to the stomach. The regular, consistent, branching patterns of these gutters are conspicuous in vesicles of the everted cardiac stomach; Figure 1 shows such a pattern in a single everted vesicle, and in Figure 4 they are clearly visible in several parts of the widely spread stomach.

Horizontal sections near the oral end of the stomach reveal the intimate relationship that exists between the gutters and various categories of intrinsic fibers. Class 1 fibers are attached longitudinally along the evaginated folds that form the gutters, and the horizontal strands of the Class 2 fibers extend from the shoulders of these folds to attach again in unspecialized areas between neighboring grooves. This arrangement of the Class 2 strands makes it possible for them to maintain the gutters by puckering the wall of the stomach and creating the inwardly-directed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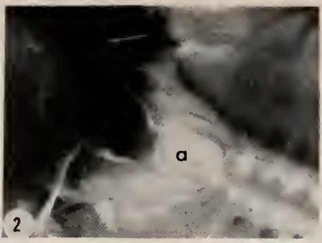
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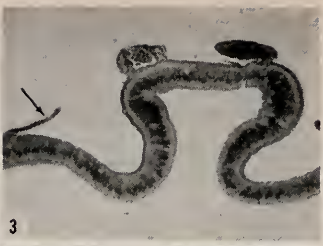
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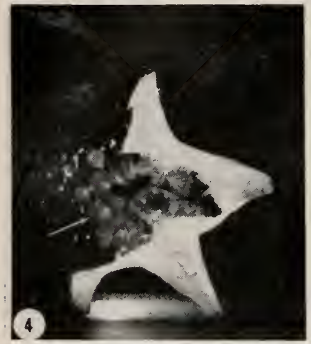
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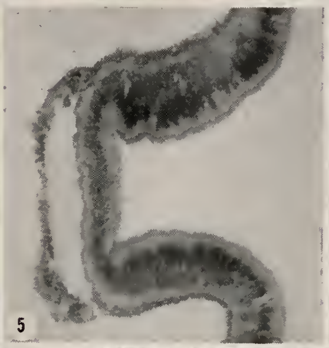
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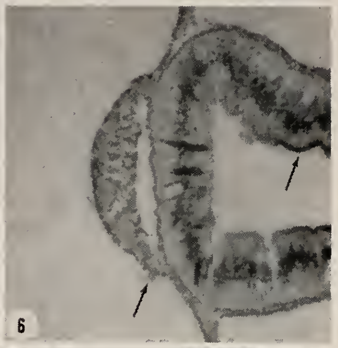
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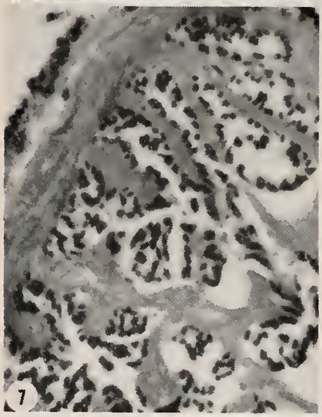
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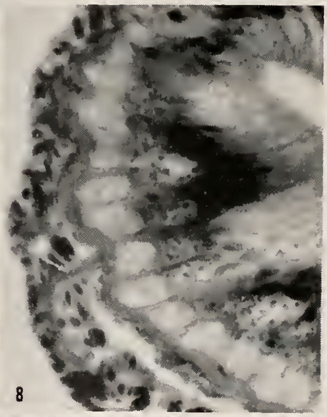
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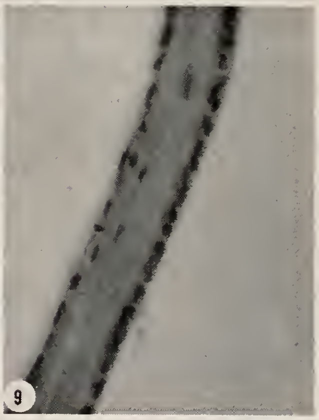
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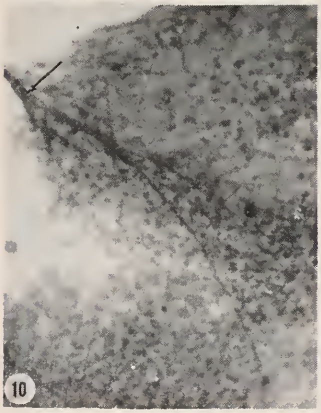
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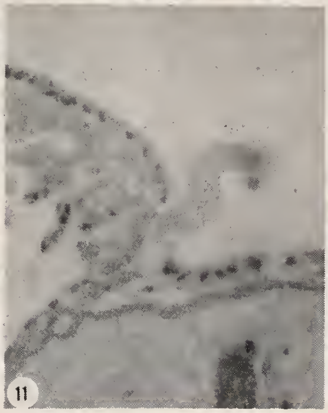
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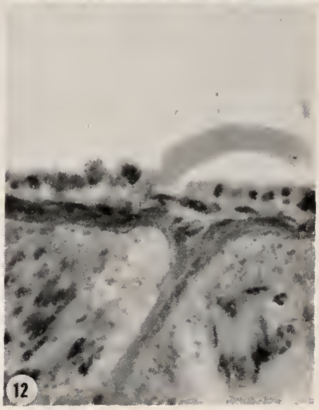
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Explanation of Figures.

Figures 1 and 2 are photographs of living preparations, and Figure 4 is a photograph of a living animal. The remaining figures on this and the following plate are photomicrographs of tissues fixed in Helly's fluid (except Figs. 7 and 20, fixed in Zenker-acetic), sectioned at 7μ, and stained as indicated. The magnifications given are approximately correct for the figures as they appear here, after enlargement and reduction.

Figure 1. Portion of the everted cardiac stomach, showing a part of the system of gutters in the stomach wall associated with Class 1 retractor fibers. Note the regular line along which the smallest branches begin, in the region termed the esophagus, and the convergence of the gutters towards the mouth (arrow). 4 ×.

Figure 2. Elements of the extrinsic retractor system in one ray, after removal of the roof of the animal with all aboral parts of the digestive tract. Note the connective-tissue girdle surrounding the stomach (arrow) ; just below this point the shortest and longest retractor strands of one side insert on the girdle, close together. Within the angle they form lie the two intermediate strands which pass onto the surface of the stomach (to left) and become continuous with Class 1 intrinsic fibers. The proximal ambulacral ossicle is indicated at *a;* this is partially covered by a perradial pouch extending from the stomach between the two short retractor strands. 4 ×.

Figure 3. Horizontal section passing through the level of confluence of two small gutters, low in the stomach; the Class 1 fibers associated with the gutters have not joined at this level. The arrow indicates a horizontal Class 2 fiber passing into the plane of the section and inserting on the stomach wall; its origin on the shoulder of the ridge nearby is not shown; *cf.* Figure 6. Phosphotungstic acid (PTA) hematoxylin; 70 ×.

Figure 4. A large individual feeding on an algal frond; the vesicles of the broadly-everted cardiac stomach have passed through holes in the frond and spread out against the glass wall of the aquarium. The arrow indicates a typical vesicle; note the great size of the stomach, and the conspicuous gutter-and fiber patterns in its vesicles. Approximately 0.25 ×. (This photograph was made by Dr. Allahverdi Farmanfarmaian of the Hopkins Marine Station and is printed with his permission.)

Figure 5. Section similar to Figure 3, higher in the stomach, with two small Class 1 fibers joining. Note the flat floor of the gutter and the general differences in the epithelium as between the floor and the sides of the gutter. PTA hematoxylin; 145 ×.

Figure 6. Horizontal section, still higher in the stomach. This section demonstrates the relationship between the three classes of intrinsic retractor fibers: a large Class 1 fiber is shown in cross-section, broadly attached by its margins to the ridge above the gutter. A pair of Class 2 fibers proceeds laterally (top and bottom, in the figure) from the shoulders of the ridge, and a coarse Class 3 fiber is shown at its origin from one side of the Class 1 strand (left arrow). Note the close relationship between the fibers of Class 1 and Class 2. In the side-wall area indicated by the right-hand arrow, note the distal deposits of basophilic granules in the epithelial cells; these are always lacking in cells lining the floor of the gutter. The dark bodies in the floor are mucous goblets. Harris' hematoxylin, light green; 145 ×.

Figure 7. Portion of a cross-section of a major Class 1 fiber, high in the stomach. At upper left, the peritoneal covering, followed by a heavy connective-tissue coat; the darkly-staining masses are shrunken bundles of muscle fibers, bound together and separated from each other by layers of connective tissue. Zenker-acetic fixation, PTA hematoxylin; 650 ×.

Figure 8. Floor of a gutter, with attachment of a Class 1 fiber; the arrow indicates a large connective-tissue bundle passing into the connective-tissue layer of the stomach wall from the retractor strand (lower left). Above this, the muscular components of the intrinsic fiber mingle with the muscular layers of the stomach wall. Lumen of the stomach to right, perivisceral coelom to left. Note the thick nerve layer, represented by the light-colored areas in arcades between the bases of the epithelial cells. PTA hematoxylin; 650 ×.

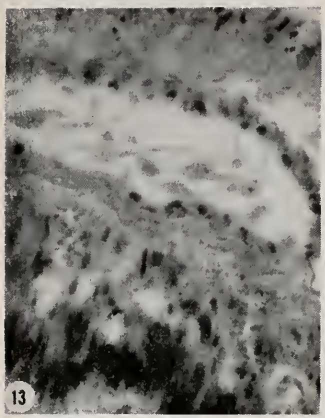
Figure 9. Longitudinal section of a Class 2 fiber, composed of parallel muscle fibers enclosed by peritoneum. Harris' hematoxylin, light green; 650 ×.

Figure 10. Tangential section of stomach wall, showing the insertion of a Class 2 fiber (arrow) and the wide distribution of its branching strands in the muscle layer of the stomach. Note that these muscle fibers are not arranged in circular and longitudinal layers but run in all directions. PTA hematoxylin; 320 ×.

Figure 11. The origin of a coarse Class 3 fiber from the attachment-point of a Class 1 fiber to the stomach wall. Note the clear, homogeneous nature of the Class 3 fiber and the shriveled coat of cytoplasm surrounding it. This fiber, turning downward towards the mouth, passes out of the section at the blurred point, where it runs alongside a second, smaller Class 3 fiber shown here in cross-section. Harris' hematoxylin, eosin; 650 ×.

Figure 12. The insertion of a coarse Class 3 fiber by branching processes penetrating the peritoneum and muscle coats to enter the connective-tissue sheet of the stomach wall. The very heavy connective-tissue layer with inward extensions is characteristic of the peristomial region, where the longest of the Class 3 fibers terminate. PTA hematoxylin; 650 ×.

Plate II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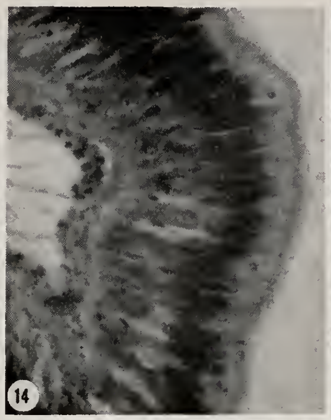
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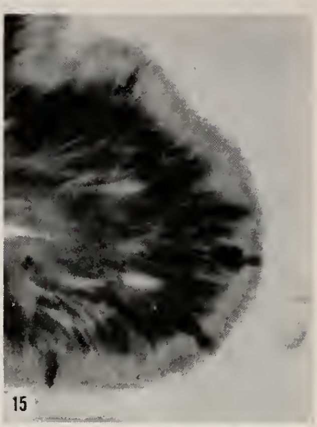
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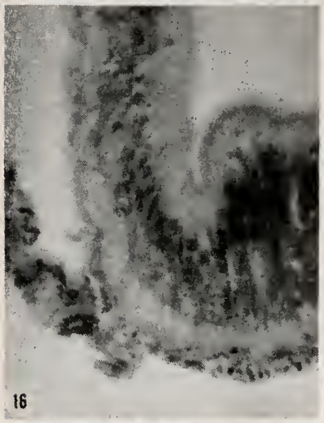
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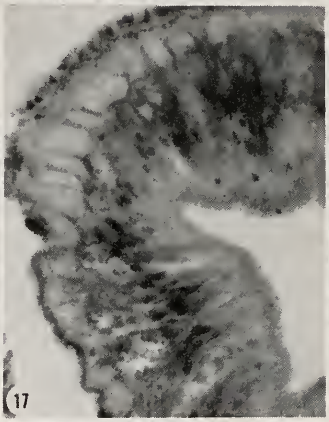
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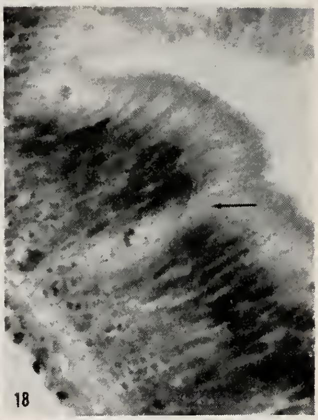
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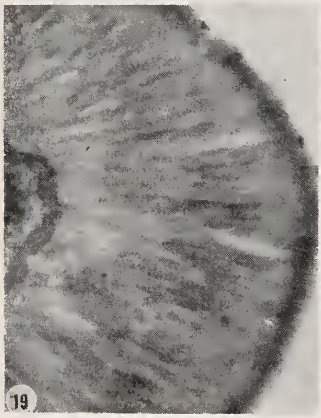
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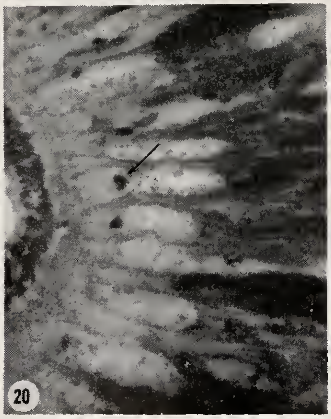
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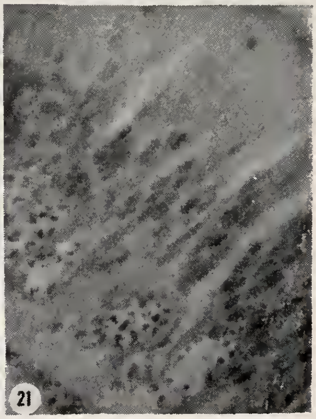
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Figure 13. A group of short Class 3 fibers lying in the coelom in a fold of the stomach wall. Note nuclei in the cytoplasmic masses attached to some. PTA hematoxylin; 650 ×.

Figure 14. Nature of the stomach wall at the lip of a gutter, in the lower part of the stomach. Note the tall epithelial cells with long, dense nuclei, characteristic of these regions

folds forming their sides. It should be noted that the preparation of this material for sectioning involved stretching and spreading segments of the stomach and pinning them to flat wax plates; even such treatment does not obliterate the gutters, which must be considered a permanent feature of the wall of the stomach.

The general histology of the stomach is typical of that repeatedly described, with minor variations, for the wall of the asteroid digestive tract (Hamann, 1885; Cuénot, 1887; Ludwig and Hamann, 1899; Chadwick, 1923; Hayashi, 1935; Anderson, 1954). A flagellated cuboidal epithelium clothes the outer surface. Just inside this lie two layers of muscle fibers, which Hamann considers as representing an outer longitudinal and an inner circular layer; in *Patiria,* muscle fibers run in all directions, forming an irregular network (Fig. 10), and it is difficult to determine which of them make up the principal circular and longitudinal strands. Inside the muscle-fiber network a connective-tissue layer is encountered. This varies in thickness, being most conspicuous in the neighborhood of Class 1 strands, which contribute fibers to it (Fig. 8). The connective-tissue layer serves also as a basement membrane, to which are attached the proximal ends of the very tall, slender, columnar epithelial cells that line the stomach and constitute the principal layer of its wall. In many areas the basal portions of these epithelial cells join

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as of the side walls of the gutters. Lumen of stomach to right, coelom to left; the random fibers lying in the coelom are small Class 3 strands. PTA hematoxylin; 650 ×.

FIGURE 15. Similar epithelium lower in the stomach, near the esophagus, showing a few secretory cells packed with coarse spherules. These are interpreted as "mulberry cells." Note the distal row of flagellary basal granules and the brush border. PTA hematoxylin; 650 ×.

FIGURE 16. Portion of the wall of a small gutter, at the zone of transition between low cells with rounded nuclei in the floor (above) and taller cells with elongate nuclei in the wall (right). Lumen of stomach at upper right, coelom to left. The irregular strand passing upward at the left is part of the Class 1 fiber associated with this gutter; note that the peritoneal layer mounts over the outside of this strand and is absent from the stomach wall beneath it. *Cf.* Figures 5, 6, and 17. PTA hematoxylin; 650 ×.

FIGURE 17. Similar section through the floor of a small gutter; lumen of stomach to right, coelom to left, with an attachment point of the associated Class 1 fiber just above left center. Note the thick nerve layer in the epithelial arcades, the small spherical nuclei high in the epithelium (amoebocyte nuclei?), and the absence of a peritoneal layer beneath the Class 1 strand. Several cysts are apparent above the nerve layer in the floor of the gutter. PTA hematoxylin; 450 ×.

FIGURE 18. Transition zone in the side of a small gutter. Note the characteristic elongated nuclei, distal differentiations of cells, a large mucous goblet (arrow) with shrunken nucleus, and numerous cysts lying above the basal nerve layer. Lumen of stomach to right, floor of gutter at top. PTA hematoxylin; 650 ×.

FIGURE 19. Typical epithelium near the lip of a gutter, showing localizations of PAS-positive material after salivary digestion. Note the conspicuous connective-tissue layer (left) and granular deposits in the distal ends of the epithelial cells. Lumen of stomach to right. Periodic-acid-Schiff, after salivary digestion, counterstained with Weigert's acid iron-chloride hematoxylin and light green; 650 ×.

FIGURE 20. Cysts between the bases of tall epithelial cells. These contain clear, refractile, homogeneous bodies; the nuclei (arrow) are the only Feulgen-positive elements associated with the cysts. Zenker-acetic, PTA hematoxylin; 650 ×.

FIGURE 21. Typical rounded-nucleus epithelium spreading from shallow gutters high in the stomach. Peritoneum at lower left, lumen of stomach at upper right. Note the long, clear mucous goblets, and the numerous cysts; compare the appearance of the contents of these cysts with those in Figures 8, 17, 18, 20. PTA hematoxylin; 650 ×.

together in bundles before inserting on the basement membrane, forming arcades of various sizes occupied by the fibers of the nerve layer (Figs. 8, 17). Like the connective-tissue sheet, the nerve layer is somewhat thicker in the bottoms of the gutters, beneath the lines of attachment of Class 1 fibers, but it never forms the thickest component of the stomach wall in *Patiria* as it does in some regions of the stomach in *Asterias forbesi* (Anderson, 1954: Fig. 10).

The epithelium lining the stomach presents no particularly remarkable features. Its typical tall columnar cells are covered distally by a brush border, and each cell bears a single long flagellum, arising from a conspicuous basal granule somewhat eccentrically placed in the distal end of the cell (Figs. 8, 18). Numerous mucous goblets are interspersed among the typical epithelial cells, with nuclei usually lying in the basal third of the cell and crowded to one side by the accumulated secretion (Figs. 6, 18). Other types of secretory cells are encountered very infrequently in the cardiac stomach proper; single cells, long and slender and containing a row or two of small secretory spherules, are occasionally found scattered at random among the cells of the general epithelium, but these are not at all common. In the region of the esophagus there is a considerable representation of secretory cells of a different type, tending to lie rather high in the epithelium and to have a somewhat bulbous appearance, packed and distended with coarse, deeply-staining spherules (Fig. 15). These I have interpreted not as endodermal zymogen cells, such as are abundant in the digestive diverticula (pyloric caeca), but as representing the so-called "mulberry cells" of the epidermis described by Cuénot (1887). Cells identical in appearance and staining behavior are numerous in the adjacent peristomial epithelium and gradually disappear in the esophagus; a very few may be encountered in the lowest part of the stomach.

A considerable degree of regional specialization, or consistent, patterned distribution of specific epithelial cell types, occurs in conjunction with the branching gutters of the stomach wall, although in *Patiria* this characteristic is never so marked as in *Asterias*. The flattened floors of the gutters are lined by comparatively low columnar cells with clear cytoplasm and small, rounded, granular nuclei (Figs. 17, 18). In the lower part of the stomach, where the gutters are narrow and deep, such cells appear only in their floors; higher in the stomach, where the gutters become broad and shallow, the columnar cells with small rounded nuclei occupy larger areas, increase in height, and constitute the most numerous class of epithelial cells (Fig. 21). The lateral walls of the deeper gutters consist of tall cells, very crowded, in which the nuclei are elongate and slender and stain densely. A narrow zone of transition marks the change from one type of epithelium to the other, just where the side walls rise from the floor (Figs. 3, 5, 16). The tall cells with dense nuclei are most numerous in the walls of the gutters; they continue over the lips of these grooves (Fig. 14) and gradually give way, in the areas between adjacent gutters, to equally tall cells with elongate but more granular and lightly-staining nuclei. Flagella are never multiple; each cell bears only one.

Mucous goblets are numerous in the floors of the gutters as well as among the taller cells in their side walls and near their lips. Small cells with spherical, granular nuclei, interpreted as amoebocytes, are commonly observed lodged between the epithelial cells, most conspicuously at levels above the epithelial-cell nuclei (Figs. 17, 18).

Although no detailed histochemical studies have been attempted, special technics demonstrate some additional characteristics in various histological components of the stomach. Glycogen appears to be very generally distributed throughout, with no notable points of concentration, as indicated by results of the periodic acid-Schiff routine. After removal, by salivary digestion, of all PAS reactivity attributable to glycogen, several sites retain strongly positive reactions. These sites include, as expected, the connective-tissue basement membrane of the stomach wall (Fig. 19) and related components of Class 1 retractor fibers, as well as the contents of mucous goblets and free mucus retained on the surface of the epithelium after release. In addition, the distal cytoplasm of virtually all the tall epithelial cells contains varying amounts of granular, finely-dispersed PAS-positive material (Fig. 19), the amount depending on the locations of the cells. Those in the side walls of narrow gutters usually contain the largest deposits; in other locations only a small amount of material just under the brush border, surrounding the basal granule of the flagellum, reacts positively. In addition to being PAS-positive, this material is basophilic (stains with Harris' hematoxylin—see Fig. 6) and exhibits a reddish metachromasia, persisting after alcoholic dehydration and mounting in resin, when stained overnight with dilute toluidine blue. These staining reactions are consistent characteristics of the distal deposits in tall cells with long nuclei; it is noteworthy that in the epithelial cells with rounded, granular nuclei, localized in the floors of deep gutters and more widely distributed in broad, shallow ones, the cytoplasm is always clear and never contains even a trace of such material. In contrast to the distal metachromatic material in the tall epithelial cells, the contents of mucous goblets, while similarly PAS-positive, lose their toluidine-blue metachromasia during alcoholic dehydration.

One further unusual feature of the cardiac stomach of *Patiria*, characteristic of all specimens examined, remains to be described, although its significance remains in doubt. In all levels of the stomach, the depths of the epithelial layer are occupied by numerous cystic growths, of various sizes. They may be small and nearly spherical, lying in the arcades between the basal processes of the epithelial cells just above the nerve layer (Figs. 16, 17, 18), or they may be much longer than their breadth and occur in such masses as to crowd the nuclei and major cytoplasmic portions of the epithelial cells into the upper half, or less, of the epithelial layer. The cysts are fully as variable in appearance as in size. They are always more or less completely filled with inclusions, which are either clear, nonstaining, refractile spherules less than 2 μ in diameter; or slightly basophilic spherules of about the same size, each of which contains a single highly basophilic granule; or somewhat larger masses or groups of very small, deeply staining flocculent or granular bodies. The cysts appear to begin, at least, as intracellular bodies; in conjunction with the smaller ones a single large nucleus can usually be demonstrated, somewhat distorted and crowded to one side so that it resembles the nucleus of an active mucous goblet (Fig. 20). This nucleus is the only body in the cysts that gives a positive Feulgen reaction; although the small, highly basophilic granules within the inclusions otherwise stain like chromatin, they are Feulgen-negative. It seems reasonable to conclude, at least tentatively, that the cysts are of parasitic origin. A detailed study of this material would probably show that the inclusions in different cysts, so obviously different in appearance, represent successive stages in the life-cycle of a sporozoan, widely distributed in *Patiria miniata* at Pacific Grove. Such a study is beyond the scope of the present investigation.

Discussion

Although the cardiac stomach of *Patiria* is very generally similar to that of *Asterias,* major differences in structural details appear when these organs are compared, as indicated briefly at several points in the preceding descriptive section. Close resemblance is not necessarily to be expected, as these genera are not closely related, belonging to different Orders; furthermore, their habits and feeding practices differ considerably. It is regrettable that comparably detailed studies have apparently not been made on *Asterina gibbosa,* a species very close to *Patiria* that has been more generally described by European investigators. It is of interest, however, to examine structural differences that exist between *Asterias* and *Patiria,* and to consider the functional differences to which they may give insight.

In view of the unusual degree and frequency of eversion of the cardiac stomach in *Patiria,* one might anticipate that provision of mechanisms for anchorage, reinforcement, and restraint of the everted vesicles, and for their rapid retraction, would be of the utmost significance. The existence of two supplementary systems of fibers here, in addition to the branching intrinsic retractor system (Class 1 fibers) found also in *Asterias,* is undoubtedly related to the functional demands of this special situation. Unfortunately, experimental evidence as to the contractility and behavior of these fibers in *Patiria* is not available; in its absence, deductions as to their probable functions may be made from the nature, histological composition, and anatomical relationships of the several fiber systems.

Class 1 strands are stout mixtures of connective-tissue and muscle bundles, anchored proximally to the extrinsic retractors (of which they are actually continuations), attaching all along their lengths by broad insertions on both the connective-tissue and muscle layers of the stomach wall, and extending to widespread terminal attachments at the extreme lower end of the cardiac stomach. These are almost certainly the principal restraining and retractor bands of the stomach; contractions in these strands would be very widely transmitted to the everted vesicles and would be most effective in compressing them to force the coelomic fluid back into the perivisceral cavity, and in drawing the collapsed folds uniformly back through the mouth. It is true that the contractility postulated by Anderson (1954) for the supposedly homologous intrinsic retractors in *Asterias* could not, after all, be experimentally demonstrated (Burnett and Anderson, 1955). In *Patiria,* however, the Class 1 strands contain a much greater complement of muscle fibers than the corresponding structures in *Asterias* and so may be considered more likely to be contractile.

Muscular contractions in Class 1 fibers may be transmitted even more broadly, in areas to which their branches do not extend, through the mediation of the Class 2 fibers. These originate in the muscle layer on ridges of the stomach wall, and the muscle fibers of which they are entirely composed may actually be traceable to the contractile components of the adjacent Class 1 strands. At their other ends, they distribute their fibers very widely in the muscle network of the stomach wall. In addition to maintaining the gutter-patterns in the lower part of the stomach, the

Class 2 fibers are of such composition and anatomical relationships that they could serve very importantly in collapsing the everted vesicles of the stomach at retraction, or simply in aiding the muscle layers of the stomach wall to resist excessive stretching in response to increased internal pressure.

In contrast, the Class 3 fibers, consisting solely of single strands of fibrous connective tissue, must function in a purely mechanical fashion. The myriads of short, parallel fibers that both begin and end in the connective-tissue layer of the stomach wall can only reinforce and restrain the extreme lower end of the stomach where it joins the peristome. The coarser, longer strands, originating in smaller numbers in the connective-tissue bundles of Class 1 fibers, must function to transmit tension from these to the lower stomach wall, in areas not reached by either Class 1 or Class 2 branches.

Little can be said concerning the functions of the extrinsic retractor harness. It is of interest, however, to note that the fan-shaped bands, of which there are two in each ray, are not of such simple construction as those in *Asterias* but are composed of groups of fibers with different relationships. The strands that continue on the stomach wall as branching Class 1 fibers are probably contractile, as their continuations almost certainly are. It is difficult to see how contractility in the strands connecting the circumferential girdle of the stomach to the ambulacral ossicles could serve any useful purpose; shortening of these strands would dilate the passageway between cardiac and pyloric stomachs and perhaps depress the floor of the pyloric stomach, but the contribution of such an action to the mechanism of eversion or retraction of the stomach is difficult to evaluate. At any rate, until experimental evidence as to the possible differential behavior of these various strands is available, further speculation is fruitless.

It has been remarked that regional specialization of the tissue components of the stomach wall is much less pronounced in *Patiria* than in *Asterias,* although the gutter-patterns with which this is associated are about equally well developed in the two forms. It is particularly noteworthy that the ridges between gutters in *Patiria* are not clothed with tall cells containing huge, densely-staining, cigar-shaped nuclei and bearing multiple flagella so characteristic of such regions in *Asterias.* A relatively small number of cells localized here in *Patiria* do contain long, comparatively dense nuclei, but the difference between these and their counterparts in *Asterias* is striking. If such cells represent sensory receptors, as Smith (1937) suggested for *Marthasterias glacialis,* then *Asterias* is obviously better supplied with gastric sense organs than is *Patiria.* Yet the stomachs of the two forms are apparently equally delicate and susceptible to injury and ought to be equally sensitive to stimuli from hazardous situations during eversion. The observed differences may simply be related to differences in the general characteristics of forms in the two Orders to which these species belong; or it may be that different, as-yet-unrecognized cells in the stomach of *Patiria* are functioning as sensory receptors. On the other hand, it may be suggested that the observed differences reflect the marked contrast in feeding habits exhibited by *Asterias* and *Patiria.* Although the stomach of *Asterias* is less frequently and less broadly everted than that of *Patiria* and hence perhaps not so often exposed to the general vicissitudes of the external environment, it is very often insinuated through the minute gape between the shells of living bivalves (Burnett, 1955; Lavoie and Holz, 1955; Lavoie, 1956) and must be provided with patches of specialized sensory epithelium for its guidance and protection. Feder (1955) has reported similar behavior in the stomach of *Pisaster ochraceus* attacking clams and oysters; and in his experiments with *Evasterias troschelii,* Christensen (1957) found that although the tube-feet of this species might often be trapped and cut off between the closing valves of an imitation clam, folds of the cardiac stomach never were. Patches of specialized epithelium closely resembling those found in the stomach of *Asterias* are very well developed also in the cardiac stomachs of *Pisaster ochraceus* and *Pycnopodia helianthoides* (personal observations, unpublished); I have no information on *Evasterias,* but the occurrence of such "sensory" patches in the stomachs of *Asterias, Marthasterias, Pisaster,* and *Pycnopodia* suggests that this may be a common characteristic of members of the Family Asteriidae, related to their habit of inserting the everted cardiac stomach into living bivalves. According to my observations, the stomach of *Patiria* operates in no such sophisticated manner; it is merely everted as broadly as possible and wrapped about whatever objects of food are available, or perhaps used as a flagellary-mucous feeding organ.

Experiments using sea-water suspensions of Congo-red-stained yeast cells or India ink to trace currents over the surface of the stomach reveal that the gutters do not, as might have been expected, serve to conduct food-bearing currents upward into the stomach. Such currents as do appear in the vicinity of the grooves are directed in such a way as to move material from the depths of the grooves upward over their lateral lips and thence away from the surface of the epithelium; there appear to be no longitudinal currents, either upward or downward, in the gutters themselves. The action of flagella on the ridges between gutters does produce currents carrying particles upward into the stomach. These observations are essentially in agreement with those reported for *Asterias forbesi* (Anderson, 1954).

Digestion of food materials of all kinds surrounded by the everted cardiac stomach or drawn into its cavity after retraction is rapid and complete. A small specimen of *Patiria* (radius about 2 cm.), trapped against the glass of an aquarium by a large individual (radius about 5 cm.) and enveloped in the folds of its everted stomach, was reduced within a day and a half to a small heap of dissociated skeletal ossicles; examples of this kind could be multiplied indefinitely, all testifying to the powerful nature of the digestive juices acting in the cardiac stomach. In view of this, the extreme paucity of anything resembling zymogen cells in the epithelium of the cardiac stomach is surprising and raises the suspicion that perhaps digestive enzymes are being produced by cells in the stomach lining that do not resemble those customarily identified as the sites of enzyme production in starfishes (Anderson, 1953). An alternative explanation, more in line with classical interpretations of asteroid digestive processes, would hold that the stomach itself produces no enzymes but serves only as the organ which envelopes the food and mixes it with enzymes brought to it from the myriads of typical zymogen cells in the pyloric caeca.

The true state of affairs with regard to these questions was determined by simple experiment. All 5 pairs of pyloric caeca were operatively removed from a large specimen without interfering with cardiac stomach, pyloric stomach, or other parts of the digestive tract; the specimen was then allowed to recover for a period of 8 weeks. Well before the end of this time the integrity of the body wall had been restored by healing of the incisions, and with it the ability to evert the cardiac stomach returned. After 8 weeks, the operated specimen was offered a large, distinctively-colored piece of liver and ovary from a snail, which it readily ingested. As a control, a similar piece of snail tissue was fed to a normal starfish of the same size. After 24 hours, the cardiac stomach of the control animal contained only a semifluid, disorganized *brei*, while the piece of snail tissue in the stomach of the operated starfish remained intact and in fact retained its original color. Dissection of this specimen revealed that regeneration of the pyloric caeca had begun but had advanced only to the extent of producing short, simple, tubular rudiments with none of the highly specialized features characteristic of the normal organs, and so presumably without any considerable population of zymogen cells. This experiment demonstrates that the stomach of *Patiria*, which displays an almost complete lack of granular secretory cells, does not secrete digestive enzymes, or at least does not produce them in quantities sufficient to bring about normal digestion. The pyloric caeca, which contain very large numbers of granule-filled cells interpreted as zymogen cells, are obviously necessary for the production of digestive enzymes; in the absence of these organs, digestion does not occur. The enzymatic secretions which they normally produce are carried to the cardiac stomach by flagellary currents, described for this species by Irving (1924), forming a part of the regular circulation that also brings the products of digestion from the stomach into the pyloric caeca.

Many other aspects of structure and function in the digestive system of starfishes await elucidation; detailed anatomical and histological studies, such as those reported here for a limited portion of the alimentary system in a single species, still remain to be done for a majority of even the commonest asteroids. Such studies, continued and broadened, will make possible meaningful comparisons between closely and distantly related species, and between species of diverse habits and ways of life. Most importantly, detailed and accurate anatomical studies serve as a basis for correlation and interpretation of the results of experimental physiological studies which can, in turn, furnish explanations for anatomical details.

Summary and Conclusions

The large and voluminous cardiac stomach of *Patiria miniata*, frequently and very extensively everted in the normal feeding behavior of this omnivorous intertidal starfish, is provided with an unusually elaborate retractor harness. As in *Asterias*, this consists of an extrinsic portion extending from the ambulacral ossicles to the stomach, and an intrinsic portion spreading over the stomach in a regular pattern of branching strands, here designated Class 1 fibers. A second system consists of small, muscular strands extending horizontally in the region of the lower Class 1 branches and inserting on the muscle layer of the stomach; these have been termed Class 2 fibers. Still a third set, composed of single strands of connective tissue, arise either from Class 1 fibers or from the connective-tissue layers of the wall of the stomach, take a short, straight, longitudinal course downward, and insert again on the connective-tissue sheet in the stomach wall. These Class 3 strands occur in large numbers, limited to the extreme lower end of the stomach. It is assumed that all these accessory components of the retractor harness have arisen in connection with special problems presented by the size and mode of operation of the cardiac stomach; the several systems of strands, varying in composition and in anatomical relationships, undoubtedly perform specific, different functions in reinforcing, restraining, and retracting the spreading vesicles of the cardiac stomach.

The wall of the stomach has essentially the same histological composition as that usually found in the asteroid digestive tract, with connective-tissue and nerve layers showing localized thickenings in the vicinity of Class 1 fibers. These strands are also accompanied by correspondingly branched, specifically distributed patterns of folds and ridges forming gutters in the stomach wall. Consistent patterns of cell localization in the epithelium lining the stomach are related to the gutters; comparatively low cells with rounded nuclei line their floors, while their side walls are clothed by taller cells with long, dense nuclei. Mucous goblets are common, but there are practically no granular secretory cells. The regional specializations in the distribution of epithelial-cell types in *Patiria* are much less marked than in *Asterias;* in particular, *Patiria* lacks the conspicuous patches of tall cells with huge, cigar-shaped nuclei and multiple flagella (thought to be sensory receptors) localized between the gutters in *Asterias.* This may indicate that the cardiac stomach of *Patiria* is less sensitive to external stimuli than that of *Asterias;* possible explanations for this may involve differences in feeding habits and the behavior of the stomach between these two types of starfishes.

The cardiac stomach of *Patiria* lacks zymogen cells almost completely; this is surprising in view of the rapidity and versatility of the digestive activities carried on by this organ. It has been experimentally demonstrated, however, that individuals operatively deprived of their pyloric caeca are unable to digest food; although they ingest it normally and hold it in the cardiac stomach, food remains intact for at least 24 hours, a period sufficient for almost complete breakdown of similar food in the stomach of a normal animal. It is concluded that the myriads of granular secretory cells in the pyloric caeca, usually interpreted as zymogen cells, are the source of the digestive enzymes acting to bring about normal digestion of food in the cardiac stomach.

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THE BREEDING SEASON OF THE BRACHIOPOD, LINGULA UNGUIS (L.)

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Yatsu (1902) believed that the breeding season of *Lingula unguis* lasted from mid-July to the end of August in Misaki, Japan, since he could not find the larvae at any other time of the year. Kume (1956) confirmed Yatsu's observation, and also found that the peak of breeding occurred during the first half of August. Sewell (1912) found several larvae in December and February in the plankton off the south coast of Burma. He attributed this (p. 90) to "either a local peculiarity or possibly to the existence of two breeding seasons during the year, one in the summer months July and August, and a second from December to February." Ashworth (1915) obtained larvae in the southern part of the Red Sea in June and October, and in the Indian Ocean in October. He also found a larva in Annandale's plankton sample obtained in the Strait of Bab-el-Mandeb in March. This led him to believe that in the southern part of the Red Sea a succession of spawnings extended at least over the period from the beginning of March to the early part of September.

Yatsu (1902) observed the spawning of *Lingula unguis* in captivity on several occasions in July and August. He noted that spermatozoa and ova were discharged forcibly through the median setal tube. Kume (1956) observed that in *L. unguis* spawning in the laboratory occurred twice daily, at sunrise and after sunset.

In the present study plankton samples were collected to determine the occurrence of the larvae of *Lingula*. The spawning behavior of adult female specimens in the laboratory was also studied.

MATERIALS AND METHODS

The plankton hauls were made with a fine tow-net at a depth one foot below the surface off the north coast of Singapore Island. On each occasion 4–10 hauls of 10 minutes each were made, more hauls being made under favorable weather conditions. The *Lingula* larvae from the plankton samples were isolated and classified according to the number of pairs of cirri. In this study a larva with "n" pairs of cirri and a pair of buds, the length of each of which has equalled the diameter of the base of the median tentacle, is assigned to the stage of "n + 1" pairs of cirri (abbreviated to n + 1 p.c. stage). Although the hauls were made 1–2 miles from a good bed of *Lingula unguis*, it was not possible to assign the larvae to this species with certainty.

Specimens of *Lingula unguis* for the laboratory study of spawning behavior were obtained from the north coast of Singapore Island. A plot of muddy sand with the greatest concentration of burrows was chosen, and from it all the available specimens were dug out with a shovel during ebb-tide. The area worked roughly amounted to 5–15 square meters per trip. In the laboratory every 25 specimens were laid on their dorsal or ventral side in a rectangular tray, 30 cm. by 45 cm., under 4 cm. of natural sea water renewed once a day. These trays were stacked on shelves in a darkened room maintained at a temperature of 18–20° C. Every morning they were examined with the light of a lamp for spawned ova. Specimens that had spawned were transferred into marked petri dishes, 9–20 cm. in diameter and with a capacity of 50–400 cc. They were maintained in these dishes with daily renewal of water during the rest of the experimental period for easier estimation of the ova spawned.

Frequent attempts were also made to find the time of settlement of the larvae by searching for the young postlarvae at the *Lingula* beds along the north and east coasts of Singapore Island.

RESULTS

I. Occurrence of planktonic larvae

The larvae of *Lingula* obtained from plankton hauls during the period July, 1952–June, 1953 are tabulated according to the number of paired cirri. Dead larvae with disintegrating cirri are assigned to the column of unclassifiable specimens (Table I).

Inspection of Table I shows that (1) the larvae appeared in practically every month of the year, suggesting a continuous breeding throughout the year. This fits in with Orton's rule (1920, p. 353), "that in those parts of the sea where temperature conditions are constant or nearly constant, and where biological conditions do not vary much, that marine animals will breed continuously." (2) On most occasions the free-swimming larvae were at different stages of development. This suggests that the larvae of each catch were not the products of any single day's spawning. (3) Older larvae of 8 and 9 p.c. stages were rare. (4) Young larvae of 2 and 3 p.c. stages appeared in July, August, September, October and November of 1952 and in January, February and June of 1953. (5) The young larvae of 2 and 3 p.c. stages were captured two or three times at intervals of 5–7 days in August and September. Their occurrences did not bear any relationship to the phases of the moon.

II. Laboratory spawning of the females in Lingula unguis

Many specimens brought into the laboratory in every month of the year were found on dissection to have ripe ova. Observations of laboratory spawning are summarized in Table II, which shows that (1) spawning occurred 5–14 days after collection among specimens collected on every occasion. (2) Observation on the duration of spawning of each batch was completed only for the batch collected on July 29, 1952. Observations on other batches were discontinued after various periods. (3) The smallest specimen that spawned had a ventral-valve length of 22.6 mm. and the largest, 46.0 mm. Presumably, the females become sexually mature in this locality when they attain the former size approximately, and continue to spawn thereafter at intervals. The presence of ripe ova in many specimens exceeding 50 mm. in length seems to indicate that the spawning power is retained in old age.

TABLE I

Number of Lingula larvae from plankton samples obtained off the north coast of Singapore Island

Date of collection	Number of larvae with the following pairs of cirri									Total
	2	3	4	5	6	7	8	9	Unclassifiable	
14. 7.1952		28	2						1	31
22. 7.1952					1					1
13. 8.1952	10	5	3						2	20
19. 8.1952	20	31								51
24. 8.1952		4	81	9	1	3			1	99
31. 8.1952										0
7. 9.1952										0
14. 9.1952			2							2
21. 9.1952	4	59	21	1						85
28. 9.1952	3	14							12	29
6.10.1952										0
26.10.1952	9	38		1	6	1				55
2.11.1952										0
9.11.1952										0
16.11.1952		10	39							49
6.12.1952										0
15.12.1952				1	1				3	5
3. 1.1953	80	15	2		4	6	3			110
2. 2.1953		4							6	10
26. 3.1953									2	2
15. 4.1953										0
20. 5.1953			6	1						7
27. 5.1953				1	4	2		1		8
12. 6.1953		2		6	59	115	12		25	219
29. 6.1953						4	8	1		13
Total	126	210	156	20	76	131	23	2	52	796

TABLE II

Laboratory spawning of Lingula unguis from the north coast of Singapore Island

Date of collection	Onset of spawning (days after collection)	Total spawning days, when expt. is discontinued	Number of males and females collected	Number of spawning females	Ventral-valve length of spawning females (mm.)	
					Minimum	Maximum
30. 5.1952				A few		
28. 6.1952	6	74	159	55	23.9	41.0
14. 7.1952	8	95	134	6	30.3	39.1
29. 7.1952	5	188	293	57	22.6	46.0
28. 8.1952	5	78	275	28	23.4	43.2
13. 9.1952	14	52	93	11	27.5	37.1
6.10.1952	12	82		11	27.0	42.3
5.11.1952	6	80	59	9	27.5	38.3
30.11.1952	11	59	65	9	26.7	39.0
31.12.1952	10	29	316	10	32.4	43.4
27. 2.1953				A few		

Further data on the batch collected on July 29, 1952 are summarized in Figure 1, which shows that (1) during a period of 6 months or so, there were some specimens spawning each week. (2) The most intensive spawning occurred between the seventh and thirteenth week of captivity when from 109–135 cases of spawning per week were observed. (3) Cases of intensive spawning when several thousand ova were extruded by a female per day occurred during the first half of the observational period.

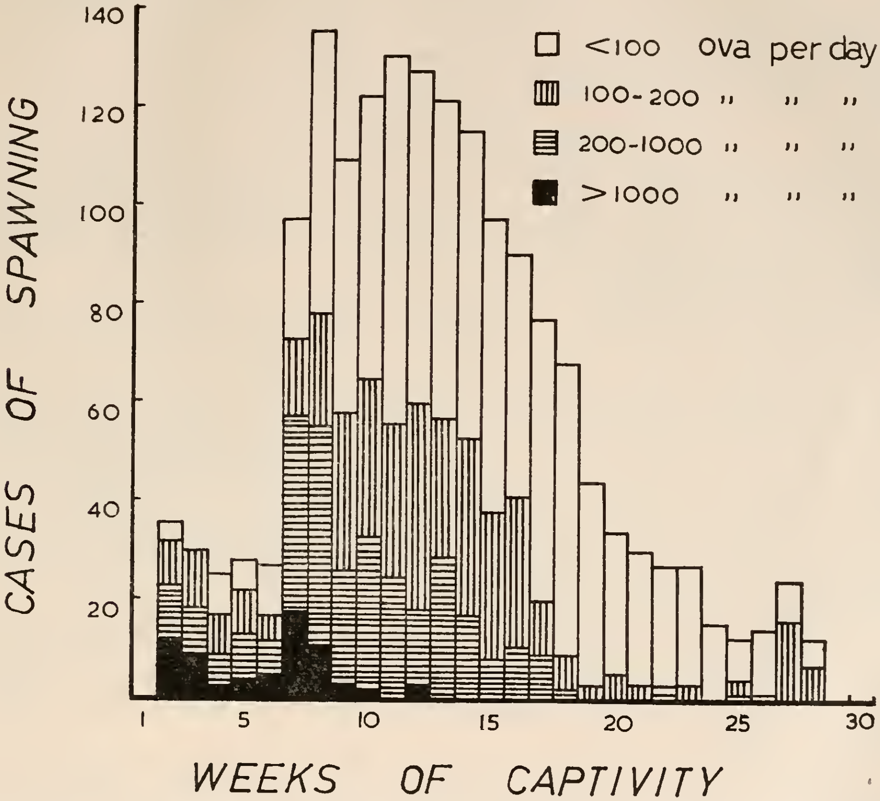

FIGURE 1. *Lingula unguis.* Number of cases of laboratory spawning per week during captivity. These cases were compiled from daily records of fifty-seven spawning females collected on July 29, 1952 from the north coast of Singapore Island.

The spawning behavior of 5 females from among the more consistent spawners of the July 29, 1952 batch reveals that (1) all the ova of an individual were not extruded at once. (2) Spawning in a female occurred in a series of bursts separated by rest intervals. (3) The first burst was usually the most intensive and consisted of 1–23 days of spawning. (4) Other bursts of lower intensity followed at irregular intervals over a period of 2–3 months. (5) The laboratory spawning did not have any relationship to the phases of the moon or the tides.

Spawning occurred both day and night. The ova were forcibly squirted out of the central exhalant setal tube, as Yatsu (1902) had previously observed. On a few occasions these came out in the accessory exhalant currents found midway along the lateral gape of the animal. In 24 hours a spawning female produced from half a dozen to about 3000 ova. When only a few ova were spawned, these were found singly in the dish. However, when a few hundred or more ova were extruded, they were coated liberally with mucus which enabled them to stick together and form big sheets or clumps, as Yatsu (1902) had noted.

The newly spawned ovum was spherical in shape. The mean of 62 ova was $95.55\,\mu \pm 4.60\,\mu$. Each was enclosed in a prominent vitelline membrane $3\,\mu$ in thickness. The nucleus was visible in the living ovum. After some time the unfertilized ovum degenerated. It gradually enlarged, presumably on imbibing water, became irregular in shape and finally disintegrated after 1–2 days in sea water.

When a transparent specimen was examined under a binocular microscope during its spawning period, the ova were found circulating in the coelomic fluid in the visceral cavity. They were also found in the coelomic fluid in the longitudinal pallial sinuses. On one occasion the pedicle of a spawning female accidentally snapped; the coelomic fluid that oozed out contained not only the usual coelomic corpuscles but also some ova.

Analysis of further data of the July 29, 1952 batch reveals that (1) large specimens produced more ova than small ones. For instance, 6 females of 41–46 mm. ventral-valve length averaged 17,250 ova per specimen during the entire observation period; 9 females, 30–40 mm. long, averaged 13,000 ova with a maximum of 17,800 ova; 3 others, all below 30 mm., averaged 4000 ova with a maximum of 7500 ova. (2) During the entire observation period of 188 days the largest specimen, 46.0 mm. long, spawned 28,600 ova in 104 days; the second largest, 43.5 mm. long, spawned 22,350 ova in 76 days. (3) A specimen, 39.8 mm. long, spawned on 125 days out of 188 days. (4) The females dissected at the end of the spawning period usually had spent ovaries.

The production of a large number of ova is presumably necessary to counteract the following possible wastages: (1) wastage of ova through fertilization taking place outside the body, when the germ cells are shed into the sea; (2) wastage of larvae due to the long pelagic larval period, which, according to Yatsu (1902), lasted 1½ months.

Observations on the laboratory spawning of female specimens suggest that the long breeding season of *Lingula* in the tropics may be due to the following: (1) all the ova were not shed at once but intermittently over a long period of time; (2) the staggering of the peaks of individual spawning in a large population; (3) the presumable tendency of the postlarvae from the different spawnings to reach spawning age at different times of the year.

The female specimens in *L. unguis* continued to spawn in the laboratory in complete isolation from the males, contrary to the hypothesis of Yatsu (1902, p. 4) that "Until the sperm is discharged the eggs even when well ripened seem to be retained within the body."

The females, even when reared singly in separate petri dishes, continued to spawn in the laboratory for several months. Crowding therefore was not necessary for the females to continue spawning, contrary to the observation of Kume (1956, p. 223) that "the frequency of shedding was greatly reduced in the vessel in which only a small number of individuals were cultured."

III. Occurrence of young postlarvae

Attempts to collect the young postlarvae were unsuccessful in the muddy *Lingula* beds along the north coast of Singapore Island. Along the east coast the beds are sandy. Here some young postlarvae with the following minimum ventral-valve lengths were obtained on the following dates: April 16, 1952, 6.5 mm.; September 24, 1952, 2.7 mm.; October 22, 1952, 3.7 mm.; November 3, 1952, 5.8 mm.; and July 3, 1953, 1.4 mm. Presumably it was only when the spatfall was considerable that the young postlarvae could be found.

Summary and Conclusions

1. The planktonic larvae of *Lingula* were found in almost every month of the year, suggesting continuous breeding off Singapore Island.
2. Several heavy spatfalls were observed in 1952, indicating several peaks of spawning during the year.
3. The spawning behavior of the female in *Lingula unguis* was described and its bearing on the continuous breeding in the tropics was suggested.
4. It was suggested that a female spawned a large quantity of ova to counteract the possible wastage of ova accompanying external fertilization and the wastage of larvae during the long pelagic larval period.

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NUCLEAR SIZES IN RANA MESONEPHROI [1]

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When Jacobj (1925) devised the now classical caryometric method there was opened up for the cytologist a whole new field in population dynamics. Jacobj demonstrated that for any given organ the nuclear population showed considerable variation in size from one individual nucleus to another. By plotting frequency distribution curves of nuclear volumes he obtained evidence to show that the volumes increased discontinuously according to a logical pattern. The peaks of the curve corresponded to nuclear volumes which when arranged in series gave the geometric progression 1:2:4:8. Nuclear class series have since been described for a wide range of both invertebrate and vertebrate tissues.

Caryometry has been used extensively in investigations of ploidy, of endomitotic growth, and of the interphasic growth of nuclei in a dividing tissue. The concept that nuclear size is a function of ploidy has proved fruitful in the study of ploidy in amphibians (*cf.* Fankhauser, 1945; Gallien, 1953). This idea was used advantageously in the interpretation of polymodal curves obtained from nuclear volume data derived from studies of the kidneys of frogs: it was indicated that polysomaty may occur in this organ (Dawson, 1948; Schreiber and Melucci, 1949). Polysomaty or endopolyploidy is understood in this paper as that condition existing in a normal diploid somatic tissue in which there is a certain percentage of polyploid cells and/or polytene chromosomes. Furthermore, the concept that nuclear size is a function of chromosomal reduplication has been helpful in the interpretation of data having to do with interphasic growth of nuclei in a dividing tissue. Nuclear class series indicative of a mitotic cycle have been described in *Ambystoma* larvae (Swift, 1950) and in *Rana pipiens* embryos (Sze, 1953). Both of these investigations were primarily concerned, not with relationships in size, but with the photometric determination of amounts of desoxyribose nucleic acid (DNA) in interphasic nuclei. The introduction into cytology of photometric techniques has renewed interest in the caryometric interpretations of nuclear sizes.

Nuclear size as a reflection of ploidy relates importantly to amphibian development both in regard to gross morphology and in regard to tissue differentiation (Fankhauser, 1945; Gallien, 1953). Also it has been shown in certain molds that both cell size and nuclear size changes may accompany morphogenesis (Bonner, 1957). Furthermore, it has been hypothesized that DNA may show a slight decrease with the progressive differentiation of certain *R. pipiens* tadpole tissues (Moore, 1952). Also of importance is the fact that nuclear size may be related to the degree of functional activity of the cells in question. For instance, spinal cord cells of *R. temporaria* show a decrease in volume following narcosis (Krantz, 1947). On the other hand, nuclear size may increase as a result, not of chromosomal increase, but as a result of protein synthesis in the nucleus. This has been shown to be true for certain insect and mammalian tissues by Schrader and Leuchtenberger (1950) and by Leuchtenberger and Schrader (1951).

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Nuclear size is, then, of considerable importance in the investigations of some of the most fundamental biological problems. The concepts and principles discussed here have not been extensively applied to nuclear size relationships of specific organs during the metamorphic stages of amphibians. Investigators have concentrated mainly on embryos before metamorphosis and on adult tissues. This paper is concerned with these concepts and principles by way of the study of the mesonephroi of *Rana sylvatica* during metamorphosis by the use of the classical methodology of caryometry.

Materials and Methods

Two clutches of *Rana sylvatica* eggs were collected in the field and were allowed to develop in the laboratory throughout an eighty-day period. The metamorphic stages selected for study at arbitrary time intervals were identified by means of the criteria established by Taylor and Kollros (1946) for *R. pipiens*. Some of the tadpoles were carried through metamorphosis to young adulthood. Table I gives both stage and age for all animals used in this investigation. Immediately after the staging of the tadpole the body cavity was opened and the animal was immersed in Zenker-formol for fixation. After fixation the mesonephroi were removed, embedded in paraffin and sectioned either transversely or longitudinally at 6 micra. All sections were stained by the Feulgen technique after Stowell (1945).

Only the center sections of a kidney from each of the 63 animals were examined. From these sections an average of 470 camera lucida outline drawings of nuclei from the convoluted tubules were made at a magnification of 1350 × diameters. The selection of nuclei to be drawn was random. However, it was necessary to set up certain criteria as a basis for selection. The first criterion was established in the following manner. In order not to cause marked distortion in the data the outline drawing of a nucleus was made at the focus showing the greatest nuclear diameter. If the nucleus is sectioned it is impossible to determine whether or not the greatest diameter lies in the section of tissue studied or in the next succeeding section. Therefore, nuclei lying in the two cut surfaces had to be eliminated on this basis. If it was possible to focus on the tissue at a level above and at a level below the nucleus selected, then it was certain that the entire nucleus was in the section. In establishing the second criterion, the long and short diameters were determined in mm. for each drawing. From these data the nuclear volumes were calculated according to the formula for the volume of an ellipsoid. It is a fact that this volume is not often used in similar studies of biologic material because of the difficulty of determining a diameter in the third dimension. This is practically impossible in sectioned material. However, in the kidneys of *R. sylvatica* used in this study the nuclei appear more or less spherical both in cross-section and in longitudinal section. On the basis of observation, therefore, it was concluded that most of the nuclei were not markedly flattened. Any nucleus that was definitely elliptical from surface view was eliminated. Also, any nucleus that appeared obviously thin on focusing through it was also eliminated. This device is, of course, arbitrary and subjective and does not remove the difficulty of the third diameter.

TABLE I

The table shows the modal volumes in mm.^{3} for the peak classes, and the ratios $\times\ x/766$ mm.^{3} where x is any one of the modal volumes and where 766 mm.^{3} is the lowest volume in the table

Stage	Class I nuclei	Intermediate class	Class II nuclei	Age in days
3	949(1.2)	1288(1.7)		18
4		1310(1.7)	1762.5(2.3)	24
		1290(1.7)		
5		1069(1.4)		22
		1226.5(1.6)		
		1075(1.4)		
6	766(1.0)	1105(1.4)	1378(1.8)	32
7		1225(1.6)	1364.5(1.8)	39
		1114(1.4)		
8		1231(1.6)	1400(1.8)	32
			1363(1.8)	
9		1066(1.5)	1369(1.8)	39
			1358.5(1.8)	
10		1270(1.7)	1500(1.9)	37
		1229(1.6)		
11–12		1130(1.5)	1511.5(2.0)	36
		1028.75(1.3)		
13–15	952(1.2)	1143(1.5)		36
		1061(1.4)		
16–17		1248(1.6)	1513(2.0)	39
		1138(1.5)		
18		1152(1.5)		43
		1294(1.7)		
		1109.5(1.4)		
19			1375(1.8)	45
			1369(1.8)	
			1355(1.8)	
20		1246(1.6)	1376.5(1.8)	47
			1355.5(1.8)	
21		1114(1.4)	1443(1.8)	47
			1381.25(1.8)	
22			1512(2.0)	50
			1926(2.5)	
			1811.5(2.3)	
23		1054(1.4)		57
		1334(1.7)		
		1300(1.7)		
24		1320(1.7)	1384(1.8)	57
		1160(1.5)		
*25	775.5(1.0)	1087(1.4)	1519(2.0)	80
	846(1.1)	1097.5(1.4)	1522(2.0)	
	958(1.2)		1417.75(1.8)	
			1349(1.8)	
			1433.5(1.9)	

* Young adults.

In addition, there are difficulties other than those due to diametric differences. For instance, there are a number of sources of error, not the least of which are those inherent in the investigator. The eye in seeing and the hand in drawing are not always coordinated to the same degree. There is the problem of focusing on the maximum nuclear surface, the possibility of inaccuracy in measurement, the effect of the procedures on the nuclei themselves, and according to Merriam and Ris (1954) the probable sources of error in the camera lucida projection method. Further, there is the fact that the error due to method has been raised to a higher power in the formula for determining volumes. However, a search for absolute values and statistical certainty is not the purpose of this paper. Only a relative value or an approximate value is sought. No attempt has been made here at statistical analysis of the data.

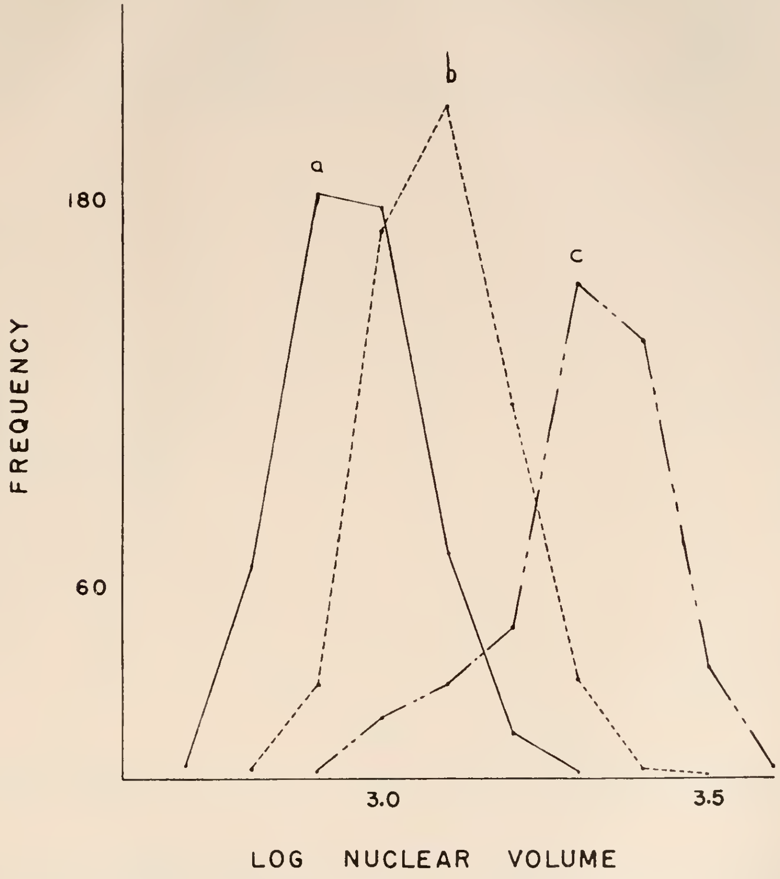

FIGURE 1. Histograms of three samples, one from each of three stages: a) stages 6, b) stage 18, c) stage 22.

After determination of the nuclear volumes, these values were grouped into frequency classes and frequency curves were drawn for each one of the 63 kidneys. The modal volume for the peak class was determined for each case and they are recorded in Table I. This table shows that the lowest modal volume (766 mm.^{3}) occurs in one of the individuals at stage 6. Using this volume for comparative purposes a ratio can be obtained for each modal volume listed. The ratios form a progression, 1 : 1.5 : 2, with nearly all intermediates between these values.

In the histograms the frequency of nuclear volumes is plotted against the class interval. Also, one third of the data were plotted by two other methods. In the one case, the long diameters of the nuclei were substituted for nuclear volumes; in the other, the logs of the nuclear volumes were plotted against frequency. The long diameters or the surface areas are frequently used as a substitute for volume when the nuclei are ellipsoidal. Both values have the advantage over volume calculations since it is not necessary to multiply the error by raising the diameters to a higher power. In this study one third of the samples were selected and frequency curves were drawn using long diameters rather than volumes. In all cases the curves correspond to those obtained in histograms in which numbers were plotted against volumes. It appears, therefore, that for the data presented here there was no great advantage of one method over the other, at least in terms of the revelation of three classes of nuclei. If, however, a curve of greater symmetry is

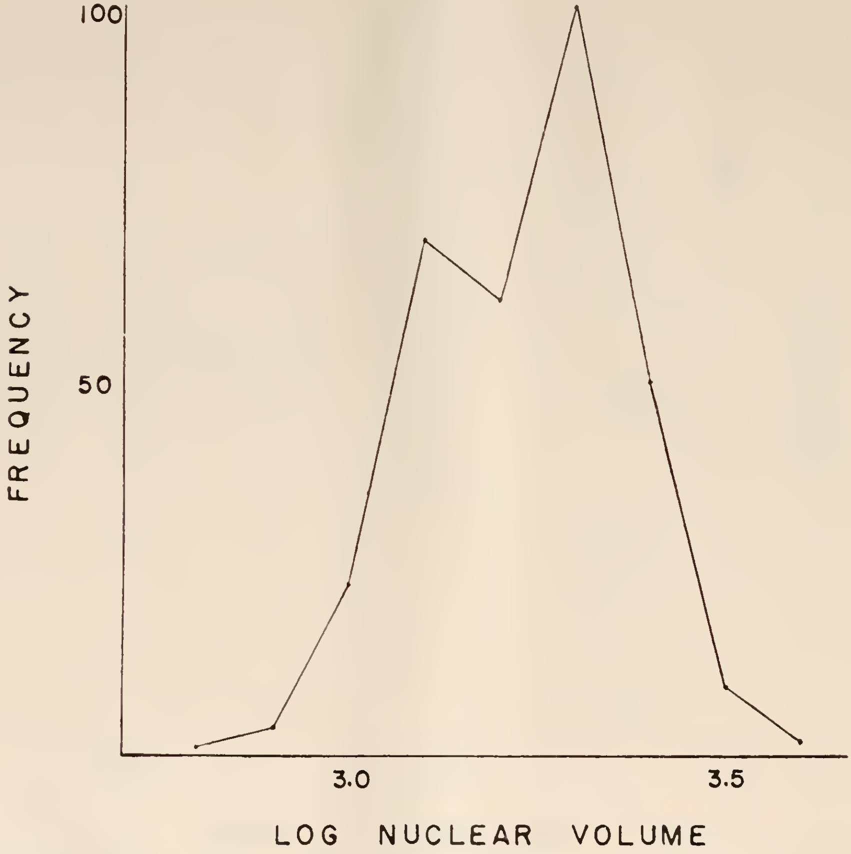

FIGURE 2. Histogram of one of the individuals at stage 24.

desired then the use of logarithms of nuclear volumes has a distinct advantage over both volume and the long diameter. The logs of nuclear volumes give a more symmetrical curve than the nuclear volume itself (Figs. 1 and 2). Bucher (1954) claimed that the logarithmic system of classification is better adapted to a mathematical analysis of the curve and that it is the only valid system from a biological and statistical viewpoint. On the other hand, it has been stated (Bonner and Eden, 1956) that a mathematical analysis of the curve is not especially helpful for comparative purposes except in those restricted cases where there is additional information about cell or nuclear growth.

Finally, the geometric mean of the nuclear volumes was calculated for each kidney for purposes of plotting the logarithmic growth curve (Fig. 3). The geometric mean is subject to all those errors inherent in the method and is, like the nuclear volume, an approximation.

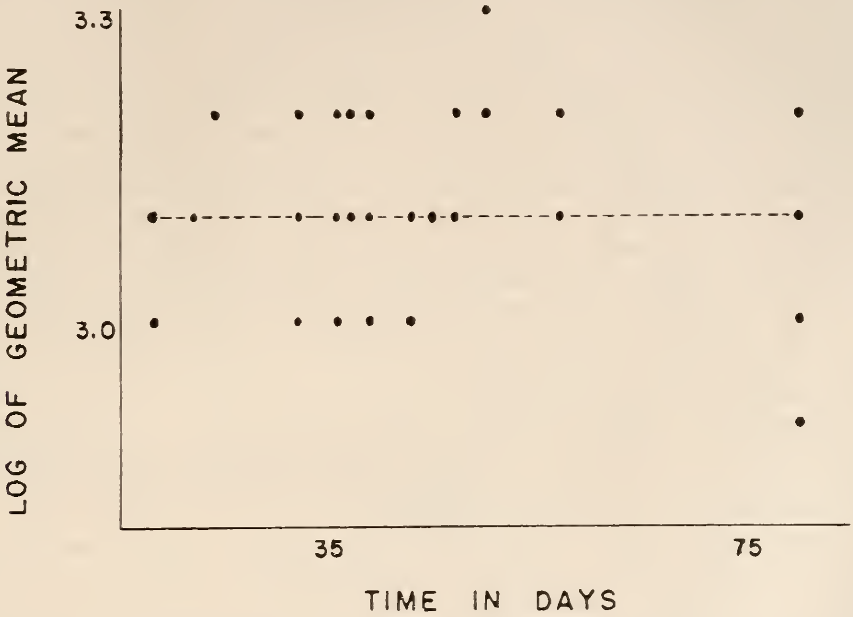

Figure 3. Logarithmic growth curve. Each point plotted represents from one to four individuals. Thus, at 80 days the 10 young adults are represented in the four points.

Observations

The frequency distribution curves of all but one of the kidneys studied show a single peak and are therefore unimodal. There is one bimodal curve (Fig. 2) in which the nuclear volumes give two peak classes. In all cases, when the modal volumes (Table I) calculated for these peaks are arranged in a series they form a progression in the ratio 1 : 1.5 : 2. Therefore, the histograms reveal two major classes and one intermediate class (Fig. 1).

In Class I the nuclei are believed to have the diploid value characteristic of post-mitotic nuclei. Following division there is, possibly, a period of interphasic growth of the chromosomes during which the nuclear volumes increase to the size of the intermediate class. This may be followed by a second period of interphasic growth during which the nuclear volume becomes twice that of the diploid nucleus. Nuclei of Class II may, therefore, be tetraploid and ready for division. All intermediate values shown in Table I are interpreted hypothetically as intermediate classes which would be expected to appear during the two interphasic growth periods. The three peak classes are shown in Figure 1 where three of the 63 histograms were selected for illustrative purposes. It is to be noted that in both Figures 1 and 2 the log nuclear volume has been substituted for nuclear volume.

Examination of Table I, with the assumptions noted above in mind, will reveal that modal volumes of Class I are rare, and that the intermediate class occurs most frequently. It is perhaps significant that whereas 30% of the young adults have modal volumes of Class I, only about 5% of the tadpoles do. The number of kidneys in the tetraploid class (Class II) are most numerous through stages 7–10, 19–22 and in the young adults. In stage 7 through 10 and in the young adults 50% of the kidneys sampled give peak classes in the tetraploid range, while in stages 19–22 approximately 83% of the samples are in this range. It appears, therefore, that the greatest periods of mitotic activity occur within the stages indicated.

The logarithmic growth curve (Fig. 3) also serves to point out some of these relationships. The growth curve is a straight line drawn through points which represent the intermediate class described above. The scatter above and below the line is due to the occurrence of samples which give peak classes in the Class I and in the Class II range. Obviously, however, there are no marked increases or decreases in growth that can be correlated with age. All nuclear classes may be found in the kidneys of tadpoles which are of the same age, just as all nuclear classes may be found in the kidneys of tadpoles in the same stage (Table I). Nevertheless, a rough correlation can be made for stages 19–22. The animals in these stages are 45–50 days old. At 45–50 days the scatter is all above the line of growth indicating, perhaps, increased mitotic activity at this time.

A single bimodal curve was revealed in one of the individuals at stage 24 (Fig. 2). The two peaks correspond to the intermediate class and to Class II nuclei. Other instances of bimodality were suggested in the histograms. But in all cases except the one they were not revealed by either of the other graphing techniques, and are considered insignificant. With the one exception all histograms regardless of the technique were unimodal. However, when nuclear volumes are used in the histograms the curves show a marked asymmetry and are skewed to the right. This marked asymmetry disappears when the logs of nuclear volumes are used in place of the nuclear volume itself.

Discussion

The commonly accepted explanation for the appearance of the different nuclear classes in the frequency distribution curve is that for some nuclei growth in size is arrested at one or another of the growth stages, resulting in an accumulation of nuclei in the stage at which growth ceased. The tissue, as a result, carries accumulations of nuclei of differing sizes which are responsible for the peaks in the curve. Nuclei with volumes in the Class II range and greater, represent tetraploid nuclei and the higher degrees of polyploidy, while those with volumes Class I–Class II represent interphasic sizes between successive mitoses. Hertwig (1939) in measuring nuclei from the early cleavage stages of mouse ova demonstrated that at each mitosis the nuclear volume was halved and he postulated that these volume changes could be correlated with similar changes in the genome. Alfert (1950), who studied cleavage stages in the mouse by photometric analysis of DNA content, reported doubling of DNA amounts in the interphase preceding mitosis. The significance of this observation derives from the fact that there is considerable evidence, obtained principally by the use of quantitative techniques, which suggests that DNA is either identical with the genic material or is at least closely associated with it. The same quantitative distributional patterns hypothesized by cytogeneticists for genic material can be directly applied to distributional patterns hypothesized for DNA. If this generalization is applied here, then a relationship must exist between nuclear volume and DNA content. Swift (1950) and Truong and Dornfeld (1955), using the photometric method for DNA determination in combination with caryometry on different animal tissues, showed that there is a definite relationship between DNA amount and nuclear size. Both volumes and DNA amounts fall into the ratio 1 : 2 : 4. In stable adult tissues (Swift, 1950, 1953; Pollister, Swift and Alfert, 1951) the DNA classes are clearly demarcated with little or no overlapping, while in actively dividing tissues there may be considerable overlapping because of the appearance of intermediate classes. The intermediate classes are due to gradual DNA synthesis during interphase. When the DNA reaches tetraploid level the nucleus enters prophase. After quantitative division of DNA at anaphase the diploid (Class I) amount of DNA is restored in the telophase nuclei. The cycle of DNA reduplication then repeats itself. Similarly, when labile tissues are studied by means of nuclear volume determinations, intermediate classes are revealed (Schreiber and Angeletti, 1940; Schreiber, 1949). In these cases the volume changes are interpreted as resulting from reduplicating phenomena in the genome.

In the kidneys of *R. sylvatica* used in this study there were no DNA determinations. Interpreting the results, however, on the basis of the above discussion it is possible to conclude that the data reveal the typical nuclear classes of a mitotic cycle, and a whole series of intermediate classes between Class I and Class II nuclei. The histograms (Figs. 1 and 2) show considerable overlapping. The overlapping is so marked that when the individuals are grouped and a combined histogram of all 63 kidneys is drawn, only one nuclear class is revealed. The modal value of this class falls approximately half way between Class I and Class II. It is generally believed that the increase in nuclear volume is rhythmic and that periods of rapid growth alternate with periods of relative inactivity. It is not possible from the results of this investigation to determine conclusively whether rhythmic growth as opposed to continuous growth is present here or not. For one thing, the degree of overlapping precludes any affirmative assumption concerning the presence of rhythmic growth. Not only are there no sharp demarcations between peaks suggesting discontinuity but there are also few positive correlations between the peak class and the stage and/or age of the animal. Secondly, the mere fact of the existence of a series of nuclear classes in a tissue does not imply that the details of individual nuclear growth are known, for normal frequency distributions may be obtained not only from actively dividing tissues but from stable tissues as well (Bonner and Eden, 1956). However, the low incidence of Class I nuclei in the mesonephros of *R. sylvatica* may be due to a period of very rapid growth immediately following mitosis, so that Class I nuclei occur only transitionally. There is the possibility that the large percentage of nuclei in the intermediate class accumulate in this class due to a decrease in nuclear activity. Nuclei from this reservoir may be released gradually into the second growth period, at the end of which there is an accumulation of Class II nuclei. Schreiber and Angeletti (1940) found that in the mitotic cycle of hepatic cells of the carp there is a rhythmic increase and decrease in nuclear volume which can be correlated with the stage of development. In the kidneys of *Rana sylvatica* there is no such clearcut correlation. All nuclear classes may be present irrespective of the stage of development or of the age of the animal (Fig. 3). The developmental pattern in the kidney does not, therefore, reflect the details of nuclear growth. However, the percentage of Class II nuclei is greatest at stages 7–10, 19–22 and in the young adults. This suggests not only the possibility of three waves of mitotic activity but also a rhythmical growth pattern.

The nuclear size increase recorded here may not be associated with the duplicating process of the genome or with an increase in DNA. It has been discovered that in a given tissue, while DNA remains constant per nucleus, the protein content and the nuclear size show corresponding increases (Alfert, 1950; Biesele, 1944; Schrader and Leuchtenberger, 1950; Leuchtenberger and Schrader, 1951). Further, the chromosomal volume may increase by protein synthesis without an accompanying morphological change in nuclear size (Biesele, 1944). Also the nuclear volume may even be reduced as a result of water loss rather than by a change in the genome (Krantz, 1947). Furthermore, differences in nuclear size may have to do with nutritional differences that do not affect chromosomal size (Montgomery, 1910). Even in a normally dividing tissue there may be a size difference due to some factor other than that which stimulates cell activity. For instance, the age of the animal may affect cell or nuclear size quite independently of a tendency toward compensatory hypertrophy (Buchner and Glinos, 1950).

In view of these considerations a number of possibilities come to mind in regard to the increase in nuclear size observed here. This increase has been attributed to mitotic activity, or to an increase in chromosomal content. It is conceivable that some of this increase is due to imbibition or to an increase in the osmotic concentration consequent upon the synthetic activity of the chromosomes. Or synthetic activity in the interphase nucleus may alter the nuclear membrane, causing osmotic changes. This may effect an increase in nuclear volume which is not exactly paralleled by an increase in the volume of the chromosome. As a result nuclear volumes may vary to such an extent that there is considerable overlapping of volume classes in frequency curves. The increase in size of a nucleus may be partially due to mechanical pressure (Teir, 1949). In addition to the factors postulated above it is known that a morphological increase in size may be due to compensatory hypertrophy following such operations as unilateral nephrectomy (Sulkin, 1949) or partial hepatectomy (Sulkin, 1943), to hormonal agents like oestrone (Salvatore, 1950; Alfert and Bern, 1951; Schreiber, 1954), or to agents which stimulate chromosomal activity such as thiouracil (Roels, 1954), alloxan (Diermeier *et al.*, 1951) and colchicine (Bucher, 1951; Fankhauser, 1952). The administration of any one of these agents may be followed by morphological changes in the nucleus, but need not necessarily be the direct cause of that change. Undoubtedly the size increase involves and is indicative of phenomena which are very complex due to the complexity of factors forming the cytoplasmic and nuclear ecology.

Some of the agents mentioned may not only be causative factors in increasing nuclear size but they may also induce polyploidy. Fankhauser (1952) by the use of colchicine induced endopolyploidy in embryos of the axolotl. Some of these embryos were originally diploid. It is generally known that polyploidy does occur in normal diploid animals. This condition, known as polysomaty or endopolyploidy, has been reported in the renal tubules of *Leptodactylus,* a South American frog, by Schreiber and Melucci (1949) and in the renal tubules of *Cyclorana,* the Australian desert frog by Dawson (1948). Polysomaty does not occur here in the kidneys of *R. sylvatica.* Twenty-six of the kidneys give modal volumes belonging to Class II. Conceivably, some of the nuclei in this peak class might be true polyploids with twice the diploid number of chromosomes rather than interphasic cells approaching a proliferative stage. There is no way of determining this, however, by the method used here. The histograms gave no peak classes at higher than the hypothetical tetraploid level. Possibly, some of the very large nuclei are octoploids. If so they are so rare that there is no great accumulation at this level and therefore the higher peaks are not obtained. In tissues showing a relatively high incidence of polyploid cells the frequency increases with age (Swartz, 1956). Polyploid cells may be absent then in the tadpole kidneys and in young adults and may appear only in older animals. However, it is now a well known fact (*cf.* Fankhauser, 1945) that polyploidy does occur in tadpole tissues. It can only be concluded, therefore, that there are few if any polyploid cells in the mesonephroi of these *R. sylvatica.* This is consistent with the general opinion that the incidence of polyploidy in kidney tissues is relatively rare, even though it is known to occur in those instances cited above.

The concept of endopolyploidy, or any concept of variation in chromosome number, renders untenable the hypothesis that for a given species the chromosome number is the same for all of the somatic cells. The "constancy hypothesis" has, therefore, been revised on the assumption that DNA is somehow related to the genic material. The constancy of DNA per chromosome set, first proposed by Boivin, Vendrely, and Vendrely (1948), and by Ris and Mirsky (1949), is supported by the work of Pollister and his school (*cf.* Pollister, Swift and Alfert, 1951). As pointed out above, increased metabolic activity in the chromosomes as shown by DNA synthesis is accompanied by an increase in nuclear size. If this increase is always proportionate to DNA increase then a constant relationship should exist between DNA content per chromosome set and nuclear size. Obviously, such a constant relationship does not exist. The phenomenon of variation in nuclear size may very well be a secondary event which may or may not be associated with DNA content. In terms of "constancy," assumptions concerning chromosome number and probable DNA content cannot always be made from nuclear volume data. There is also a certain amount of evidence which calls into question the constancy of DNA per chromosome set. Roels (1954) working with rat thyroid and Diermeier *et al.* (1951) with the liver of alloxan diabetic rats have presented evidence to indicate that the DNA content of a cell may vary with its degree of functional activity. Pasteels and Lison (1950) concluded that the DNA content of certain rat tissues is lower than the diploid value because of chromatin diminution. Though this particular evidence has been questioned (Alfert and Swift, 1953), chromatin diminution is known to occur in *Ascaris* (Boveri, 1904) and in some other forms (see the brief review of Tyler, 1955.) Also, Marshak and Marshak (1955) discuss negative DNA reactions in the *Arbacia* egg. Their evidence, however, has also been questioned (*cf.* Burgos, 1955; Marshak and Marshak, 1956). The exceptions to DNA constancy still raise a question in regard to the chemical nature of the gene, and they must be kept in mind when interpreting data having to do with nuclear volumes.

The occurrence of mitotic activity in the mesonephroi herein investigated indicates that during metamorphosis the kidney has some cells that are undifferentiated. At least physiologic inactivity in terms of renal function can be postulated for the proliferating cells. Some of the non-dividing cells, however, must be already manifesting renal activity. If so then the nuclear sizes may be indicative of a differentiating process. The fact that nuclear size and characteristic cell structure are intimately related is most dramatically demonstrated in insects. For example, in certain honeybee tissues (Merriam and Ris, 1954) the nuclear volume not only increases with age but there is a direct correlation between the nuclear size and secretory activity. Of equal importance is the fact that in certain mammalian tissues such as rat liver the increase in numbers of large nuclei closely parallels the histological and functional development of the organ (Sulkin, 1943; McKellar, 1949). A somewhat similar relationship has been demonstrated for a species of the slime mold, *Dictyostelium* (Bonner, 1957). In this mold, the stage of development and the early stages of differentiation are reflected in both the cell size and in the nuclear size which is characteristic of the particular stage. Further, the most active cells of this slime mold are located at the anterior end of a migrating sausage-shaped mass. The active cells are also the larger and are, according to Bonner, responsible for important morphogenetic effects which end in the formation of the fruiting body. Another parallel between cell size and differentiating activity may be seen in the developing sea urchin egg. It is well known that the micromeres formed during the cleavage stages of the sea urchin egg play an important role in morphogenesis in that they induce vegetal differentiation. McMaster (1955), working with *Lytechinus,* has shown by photometric determinations of DNA amounts in the cleavage cells that the lowest DNA amounts are in the micromeres. It is possible that the differentiating effects of these cells may be due to the lower DNA values. Once again, therefore, the point to be made here is that cell size is associated with a differentiating process. Progressive differentiation may also be associated with quantitative differences in DNA amounts in amphibians. Moore (1952), in working with haploid and diploid tissues of *Rana pipiens* embryos, found that the range of DNA values in the forebrain was greater in the 7-day embryos than in the 11-day embryos. She found no correlation between mitotic activity and the amount of DNA. In explaining this she discusses a possible correspondence between DNA amount and differentiation. DNA may decrease with age and with the maturation of the tissue until it reaches some more or less constant value. If this is so then nuclear volumes may also be smaller in adults than in embryos. Perhaps increased differential activity accounts for the larger percentage of Class I nuclei recorded (Table I) here for the young adults of *Rana sylvatica.*

At histological maturation differentiation may be influenced by polysomaty. Polyploid cells at the time of differentiation may return to a lower value by some process such as reduction mitosis (Huskins, 1948). The genic segregation which accompanies the reductional division may produce cells whose differentiating potential is quite different. This interpretation puts stress on ploidy as a causative factor in differentiation. In considering polyploid organisms it is known that generally they differentiate normally and effects of ploidy, such as larger nuclear or cell size, are considered secondary. Mather (1948) has insisted that duplication of the chromosome number is not the cause of differentiation. The fate of the cell, he concluded, is dependent not so much on the nucleus which in most cases is diploid in sexually reproducing animals or plants, but on a cytoplasm which is inherited from the past. The action of the nucleus is effective only because the cytoplasm has changed at each step along the way. At each division the nuclei are quantitatively and qualitatively alike but each one inherits a portion of cytoplasm, one portion of which cannot be equated with the other. If polyploidy occurs it is probably in response to the cytoplasm.

In conclusion, the difficulties of interpreting nuclear size relationships are many and varied. Not only are the techniques used for such studies possessed of their own difficulties but nuclear size itself may be altered by a number of conditions. Size changes may be physiological, mitotic, or due to different degrees of heteroploidy. If genic changes are involved then these changes may be correlated with alterations in DNA amounts. The results strongly suggest that DNA is the genic material. But the fact of the matter remains that the gene is a biological concept, an abstraction, known only in its effects. The exact relationship between the gene and DNA is still unknown. A nuclear size change, itself, may be a phenomenon occurring coincidentally with differentiation, as if there were two progressions, one of which functions as the cause or the effect of the other. Or instead, all the events involved may converge and commingle as an expression of a single phenomenon reaching peak expression in the differentiated living cell. Increases or decreases in nuclear size are morphological and physiological events which take part in this convergence.

Summary

1. The mesonephroi from 63 *Rana sylvatica* tadpoles and young adults were studied in sectioned material by means of a caryometric method for determination of nuclear volumes. Frequency histograms drawn from the data reveal three peak classes which, when arranged in series, give the progression, 1 : 1.5 : 2. These results are interpreted as being due to an interphasic growth preceding mitosis.

2. The results are discussed in terms of morphological increase and decrease in nuclear size, increase and decrease of DNA values, polyploidy, the "constancy" hypothesis, physiological activity and histological differentiation.

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THE ROLE OF ADSORPTION AND MOLECULAR MORPHOLOGY IN OLFACTION: THE CALCULATION OF OLFACTORY THRESHOLDS

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The surface of the olfactory nerve cell is folded into a number of delicate, hair-like protoplasmic filaments (Engström and Bloom, 1953; Bloom and Engström, 1952) which have long been considered to be the ultimate sensory processes of the receptor cells. Admirable photographs showing these hairs, a discussion of their possible functions and a description of the anatomy of the entire olfactory epithelium of various vertebrates are given by Le Gros Clark (1957) and by Allison (1953).

The filaments serve to give the olfactory receptors a large surface area. That this enlarged surface readily adsorbs odorants was shown by Moncrieff (1954, 1955) who made direct measurements, using a sheep's head, and found that odorants are adsorbed strongly and rapidly on the olfactory epithelium and that the process is reversible.

Neither the structure nor the exact composition of the human olfactory cell membrane is known. Hopkins (1926) examined the olfactory filaments of the frog which he found to be extensions of the olfactory cell membrane; he noted that the hairs reduce osmic acid and are disrupted by organic lipoid solvents, the proximal parts only remaining intact. The human olfactory membrane will probably resemble that of the frog and, like other nerve cells, will consist of a few layers of oriented lipid and protein molecules. This membrane is continually bathed by mucus (effectively saline) at a pH of about 7.2.

Davies and Taylor (1954) used the erythrocyte membrane as a model for that of the olfactory nerve cell, and showed that a large number of odorous substances act as accelerators of haemolysis by saponin. Moreover, for these substances, the logarithms of the olfactory thresholds (for man) are directly proportional to the logarithms of the haemolytic accelerating powers. This is shown in Figure 1. Some of these compounds which act as haemolytic accelerators have also been shown to cause a leakage of potassium ions across the red cell membrane (Davson and Danielli, 1938). This correlation lends support to theories of olfaction such as those of Ehrensvärd (1942) and Davies (1953a, 1953b) which postulate that odorant molecules must first adsorb onto the plasma membrane of the olfactory cell. Ehrensvärd's theory that the potential changes due to adsorption at this interface initiate the nerve impulses ascribes more importance to the specific polar groups of the odorant molecules than does the present work. Further experiments on potentials on the lines of those of Ehrensvärd and Cheesman (1941) would be of interest in this connection.

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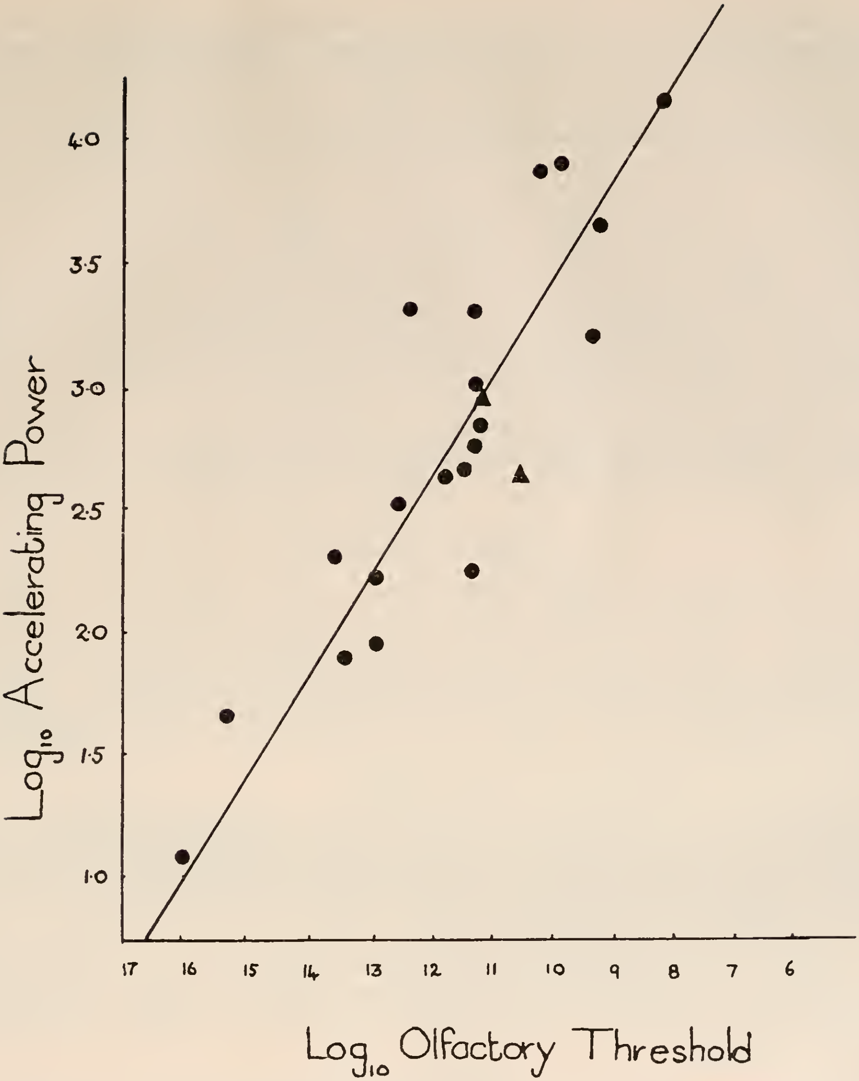

FIGURE 1. Plot of the logarithm of the haemolytic accelerating power against the logarithm of the olfactory threshold (in molecules/cc.). The equation of the line which is drawn in can be deduced from equation (4) if certain approximations are made (Davies and Taylor, 1957).

The present work, following the theory of Davies, assumes that the odorant molecules can simply dislocate the olfactory cell membrane, permitting an exchange of sodium and potassium ions across it. This exchange of ions initiates the nervous impulse as in the general theory of Hodgkin and Katz (1949).

Recently, Rideal and Taylor (1958) have shown that the logarithm of the haemolytic accelerating power of a substance is directly proportional to its free energy of adsorption at the oil-water interface; this means that the effectiveness of compounds as haemolytic accelerators depends only on the number of molecules adsorbed on the red cell surface and not, in addition, upon such factors as molecular shape or size. In olfaction, however, additional factors must be important since the slope of the line in Figure 1 is not unity and also because there is some scatter of the points about the line. This scatter may, in part, be due to the roughness with which olfactory measurements can be made, but it is also likely that the sizes, shapes and flexibilities of the penetrating molecules are important (see Davies, 1953a, 1953b). Indeed, Timmermans (1954) and Mullins (1955) have recently emphasised this point. Further, among isomers, the one with the bulkiest molecule (*i.e.*, with the most branched chains) has the lowest olfactory threshold even though it may be less strongly adsorbed at a fatty surface than the corresponding linear molecule. This applies to *n*-amyl alcohol (threshold 6.8×10^{12} molecules/cc.) and to isoamyl alcohol (threshold 6.8×10^{11} molecules/cc.) (von Skramlik, 1948) and also to the isomeric decanols; it is true for *Phormia* as well as for humans.

In this paper, the relative importance of adsorption and of molecular morphology in olfaction is assessed quantitatively, and an equation derived from which the olfactory threshold of any particular compound may be calculated.

THEORETICAL SECTION

For mathematical convenience, we assume that an olfactory cell surface is divided into n small, non-specific areas or sites, each of area α sq. cm. In order to stimulate a cell, a critical number of odorant molecules must be concentrated on one site in the cell surface. For the strongest odorant, which is assumed to be β ionone, perhaps only one molecule need occupy one of these areas. Weaker odorants, however, such as acetic acid or methanol have a less "dislocating" effect on the cell membrane and so p molecules must be concentrated simultaneously on one of the sites to cause a response.

The quantity $1/p$, then, is a measure of the "puncturing" ability of the odorant, *i.e.*, of the effectiveness of the molecule in causing the necessary ionic leakage across the cell membrane. For the strongest odorants $1/p = 1$ whilst for weaker odorants $1/p < 1$, and for water which is continually bathing the cell without causing any stimulus, $1/p = 0$.

To adsorb on the sites, the molecules must pass from the air through the aqueous (mucous) phase. This process is reversible (Moncrieff, 1955) and at equilibrium the distribution of molecules is given by (1) which is a simplified Langmuir isotherm.

$$\frac{x}{c\,d} = K_{L/A}. \tag{1}$$

Here x is the average membrane concentration of odorant molecules/sq. cm., c is the average concentration of molecules/cc. in the air, and d is the surface thickness (about 10 Å). $K_{L/A}$ is the adsorption constant for molecules passing from air to the lipid-water interface. The number of sites, N, per nerve cell containing p molecules where p is greater than the average number αx is given by Poisson's equation:

$$N = \frac{n\, e^{-\alpha x}(\alpha x)^p}{p!}, \tag{2}$$

where n is the total number of sites/cell.

At the olfactory threshold, only one site with the p adsorbed molecules will be required to stimulate the cell minimally, so that N is unity and c is the olfactory threshold. By combining equations (1) and (2) we can eliminate x and obtain a relation between $K_{L/A}$, p and the olfactory threshold. The relation in its final form is given by (3) and has been derived in full elsewhere (Davies and Taylor, 1957).

$$\log \text{O.T.} + \log K_{L/A} = \frac{-\log n}{p} + \frac{\log p!}{p} - \log \alpha d. \tag{3}$$

Calculation shows that for the weakest odorants possible, p lies between 15 and 30 and an arbitrary value of 24 is taken. For these compounds the quantity ($\log$ O.T. $+ \log K_{L/A}$) tends towards a value of 22. The compound with the lowest recorded olfactory threshold (β ionone; as listed by von Skramlik, 1948, and confirmed by the measurements of Neuhaus, 1953b) is assumed to have a p value of 1. If any more powerful odorant were discovered and assigned a p value of unity, the numerical values of the thresholds predicted would change slightly, but their relative values would not.

Application of these boundary conditions enables values of the constant $\log n$ and $\log \alpha d$ to be found; equation (3) then takes the form:

$$\log \text{O.T.} + \log K_{L/A} = \frac{-4.64}{p} + \frac{\log p!}{p} + 21.19. \tag{4}$$

Using this equation we can calculate the olfactory threshold of any substance, knowing its adsorption constant between air and the lipid-water interface and its value of p.

METHODS

The adsorption constants for molecules going from water to the oil-water interface have been determined from measurements of the lowering of the interfacial tension at the petroleum ether-water interface (Haydon and Taylor, 1959). Non-polar compounds (*e.g.*, hydrocarbons) will dissolve in the lipophilic interior of the membrane, and so for these compounds, distribution constants for molecules passing from water to the petroleum ether (bulk) phase have been used. Since we are dealing with a fatty membrane, the adsorption at the membrane water interface will be approximately equal to that at the oil-water interface.

$$K_{O/W} = K_{L/W}.$$

In fact $K_{L/W}$ will be slightly less than $K_{O/W}$ by a factor depending on the dielectric constant at the membrane surface. The distribution constant for molecules going from air to the aqueous phase has been found from the ratio of the solubility of the substance in water at 20° C. to its vapour pressure at the same temperature, or, in some instances, from the measurements of partial vapour pressures of aqueous solutions of the substances recorded in the literature.

From $K_{L/W}$ ($= K_{O/W}$) and $K_{W/A}$, the term $K_{L/A}$ ($= K_{O/A}$) required in equation (4) may be obtained directly since

$$K_{L/A} = K_{L/W} \cdot K_{W/A}.$$

Values of $\log K_{O/A}$ are listed in Table I.

TABLE I

Compound	$\log_{10} K_{O/W}$	$\log_{10} K_{W/A}$	$\log_{10} K_{O/A}$	Cross-sectional areas Å^2	
				a	b
Methanol*	1.235	4.245	5.48	20.0	12.4
Ethanol*	1.80	3.99	5.78	26.7	15.4
Propanol*	2.41	3.81	6.22	32.6	18.3
Butanol	2.85	3.69	6.54	38.0	19.2
Pentanol	3.71	3.42	7.13	43.0	20.0
Hexanol	4.39	3.18	7.57	47.8	20.0
Heptanol	4.82	2.83	7.65	52.3	20.0
Octanol	5.30	3.04	8.34	56.0	20.0
Decanol	6.94	2.04	8.98	64.6	20.0
β ionone	5.79	2.56	8.35	69.7	57.6
Piperonal	3.41	3.48	6.89	47.3	41.4
Menthol	4.505	2.015	6.52	62.8	44.8
Skatol	4.76	3.84	8.60	53.5	42.5
Xylol musk	4.58	3.96	8.54	74.0	56.2
Isoamyl alcohol	3.71	3.50	7.21	43.0	26.0
Isobutanol	2.26	3.81	6.07	38.0	24.0
Camphor	3.335	2.64	5.98	59.7	43.6
Phenol	3.04	4.66	7.70	37.9	24.1
Isoamyl acetate	3.63	1.82	5.45	52.6	30.0
Nitrobenzene	3.85	8.14	6.99	42.2	24.1
Coumarin	2.83	4.48	7.31	48.0	41.3
Pyridine*	3.25	4.78	8.03	36.3	24.0
Cycloheptadecyl lactone	5.33	3.42	8.75	89.0	50.0
Glycerol*	1.93	4.00	5.93	36.5	36.0
Cyclohexanol	3.92	4.05	7.97	45.5	27.0
Water	—	—	—	14.0	8.1
Naphthalene**	4.67	1.82	6.49	48.5	42.0
Benzene**	3.09	0.75	3.84	36.1	24.0
Cyclohexane**	3.56	−0.35	3.21	43.6	27.0
Ethane**	1.23	−0.66	0.66	24.4	15.4
n-butane**	2.40	−0.60	1.80	36.1	20.0
n-pentane**	2.97	−0.66	2.31	41.2	20.0
n-heptane**	4.15	−0.59	3.56	50.6	20.0
n-nonane**	5.33	−0.76	4.57	59.3	20.0
n-undecane**	6.52	−0.74	5.78	67.3	20.0
n-butyric acid*	3.19	6.17	9.36	38.7	20.0
n-valeric acid	3.78	5.04	8.82	43.8	20.0
Caproic acid	4.38	4.85	9.23	84.5	20.0
Oenanthic acid	4.97	4.55	9.52	53.0	20.0

Data needed to calculate olfactory thresholds for a number of odorants.

* $K_{W/A}$ calculated from published data on the partial vapour pressures of aqueous solutions of the substances.

** $K_{O/W}$ for non-polar compounds refers to passage from water to bulk of the oil phase.

The cross-sectional areas in column a have been calculated from the molecular volumes assuming the molecules to be spheres. The molecular volumes have been obtained by assuming the additivity of atomic volumes using data given in Partington (1951).

Areas in column b have been measured from models.

RESULTS AND DISCUSSION

(*a*) *The calculation of olfactory thresholds*

Since $1/p$ is a measure of the ability of an odorant molecule to "puncture" or dislocate the membrane temporarily, it is expected to be a function of molecular shape and size. These quantities are difficult to define, however, and for present

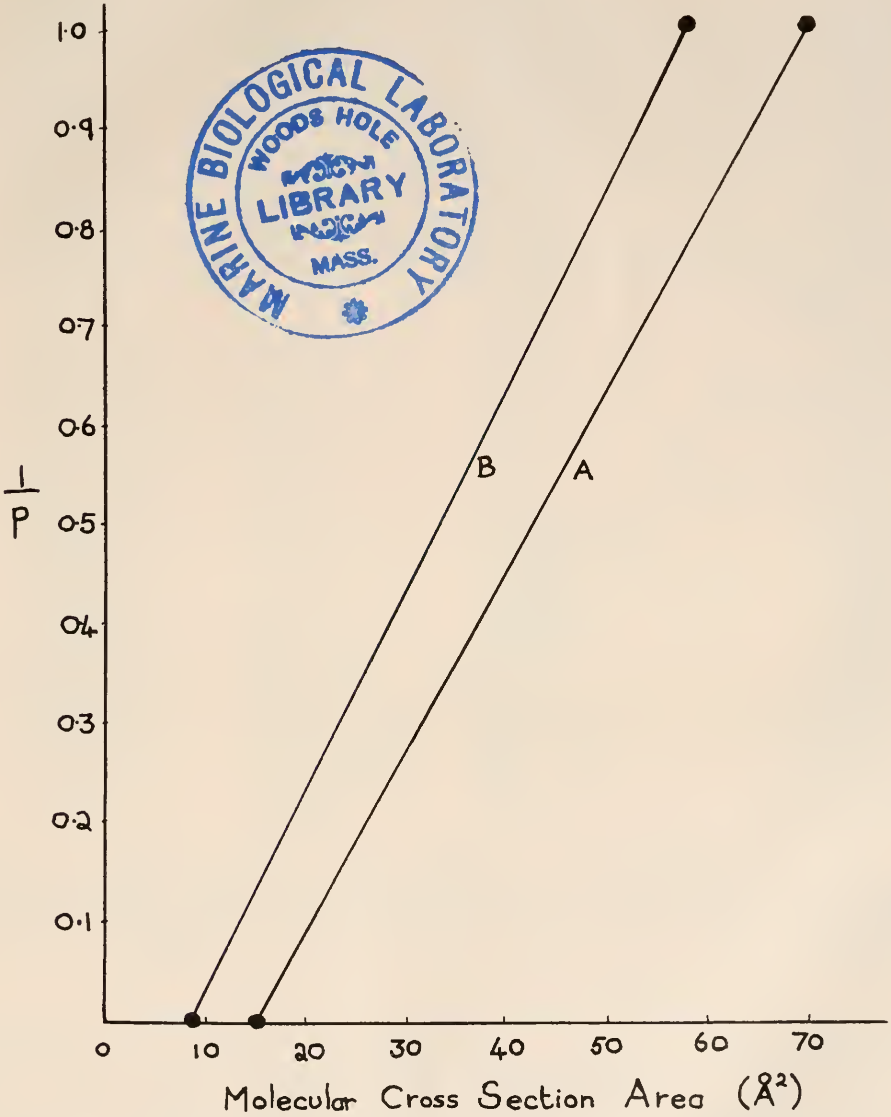

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FIGURE 2. The relationships which are assumed to hold between $1/p$ and the molecular cross-sectional areas in the evaluation of $1/p$, when (A) cross-sectional areas derived from molecular volumes are employed (molecules assumed to be spheres). (B) Measurements on models have been employed (molecules uncoiled and orientated in the membrane).

Table II

Compound	Olfactory thresholds in molecules/cc.		
	Observed	a	b
Methanol	1.10×10^{16}	8.13×10^{15}	1.15×10^{16}
Ethanol	2.44×10^{15}	6.61×10^{14}	2.04×10^{15}
Propanol	5.00×10^{13}	5.13×10^{13}	3.00×10^{14}
Butanol	8.20×10^{12}	7.94×10^{12}	1.12×10^{14}
Pentanol	6.80×10^{12}	9.78×10^{11}	2.14×10^{13}
Hexanol	6.72×10^{12}	1.32×10^{11}	7.76×10^{12}
Heptanol	9.00×10^{11}	5.25×10^{10}	6.46×10^{12}
Octanol	3.00×10^{10}	4.27×10^{9}	1.32×10^{12}
Decanol	3.63×10^{11}	1.78×10^{8}	3.02×10^{11}
β ionone	1.60×10^{8}	1.60×10^{8}	1.60×10^{8}
Piperonal	2.00×10^{11}	7.08×10^{11}	3.39×10^{11}
Menthol	2.00×10^{11}	8.32×10^{11}	3.31×10^{11}
Skatol	1.80×10^{9}	4.07×10^{9}	4.47×10^{9}
Xylol musk	2.10×10^{9}	4.47×10^{7}	1.58×10^{8}
Isoamyl alcohol	6.80×10^{11}	8.13×10^{11}	3.63×10^{12}
Isobutanol	8.20×10^{12}	2.34×10^{12}	8.13×10^{13}
Camphor	5.00×10^{12}	4.90×10^{11}	1.45×10^{12}
Phenol	7.90×10^{12}	5.37×10^{11}	1.82×10^{12}
Isoamyl acetate	1.82×10^{14}	6.92×10^{12}	8.51×10^{13}
Nitrobenzene	2.00×10^{11}	1.32×10^{12}	9.55×10^{12}
Coumarin	2.10×10^{11}	2.40×10^{11}	1.32×10^{11}
Pyridine	3.10×10^{11}	3.63×10^{11}	8.91×10^{11}
Cycloheptadecyl lactone	1.75×10^{10}	2.76×10^{6}	5.50×10^{8}
Naphthalene	2.60×10^{12}	1.45×10^{12}	3.09×10^{13}
Benzene	4.00×10^{13}	5.75×10^{15}	1.38×10^{16}
Cyclohexane	1.82×10^{15}	6.46×10^{15}	2.88×10^{16}
Ethane	1.30×10^{19}	1.70×10^{20}	6.31×10^{19}
n-butane	7.11×10^{16}	6.31×10^{17}	4.57×10^{18}
n-pentane	2.73×10^{16}	7.59×10^{16}	1.41×10^{18}
n-heptane	1.32×10^{16}	8.51×10^{14}	7.94×10^{16}
n-nonane	4.87×10^{15}	1.32×10^{13}	7.76×10^{16}
n-undecane	1.35×10^{15}	1.15×10^{11}	4.79×10^{14}
n-butyric acid	1.4×10^{11}	1.05×10^{10}	1.26×10^{11}
n-valeric acid	1.2×10^{11}	1.55×10^{10}	4.37×10^{11}
Caproic acid	1.2×10^{12}	2.63×10^{9}	1.70×10^{11}
Oenanthic acid	1.35×10^{13}	5.13×10^{8}	8.71×10^{10}

Observed and calculated olfactory thresholds. The observed values have been taken from those listed in the publications of von Skramlik (1948), Backman (1917), Morimura (1934) and Mullins (1955). The calculated values listed in column a have been obtained assuming that the molecules are spherical. Those in column b assume that the odorant molecules are uncoiled and orientated.

purposes the cross-sectional areas of the molecules are employed. These have been calculated in two ways: those obtained from the molecular volumes are derived assuming that the molecules are spherical; for straight chain organic molecules this means that the chains must be coiled up. This is likely to be so when the molecules are in water but is unlikely in a fatty membrane. In measurements taken from models, therefore, it is assumed that the molecules adsorbed in the membrane are orientated with the hydrocarbon chains completely uncoiled;

in a homologous series the cross-sectional area is thus constant for the higher members.

If it is assumed that the dislocating power $1/p$ varies linearly with the molecular cross-sectional area, then values of $1/p$ can be calculated, since for β ionone $1/p = 1$ and for water $1/p = 0$ (Fig. 2). Values of the areas used for this purpose are listed in Table I.

From $1/p$ and $\log K_{O/A}$ olfactory thresholds can be calculated using (4); values thus obtained are included in Table II. In Figures 3 and 4 the observed and calculated values are compared, assuming respectively that the molecules are spherical and that they are unfolded and orientated. It is seen that there is good agreement between calculated and observed values. This encourages us to believe that the "puncturing theory" of olfaction is essentially correct and that, to cause a stimulus, more small molecules must be packed into a given area of membrane surface than large ones.

In general, and for the aliphatic hydrocarbons and alcohols in particular, the observed thresholds tally much better with those calculated assuming that the

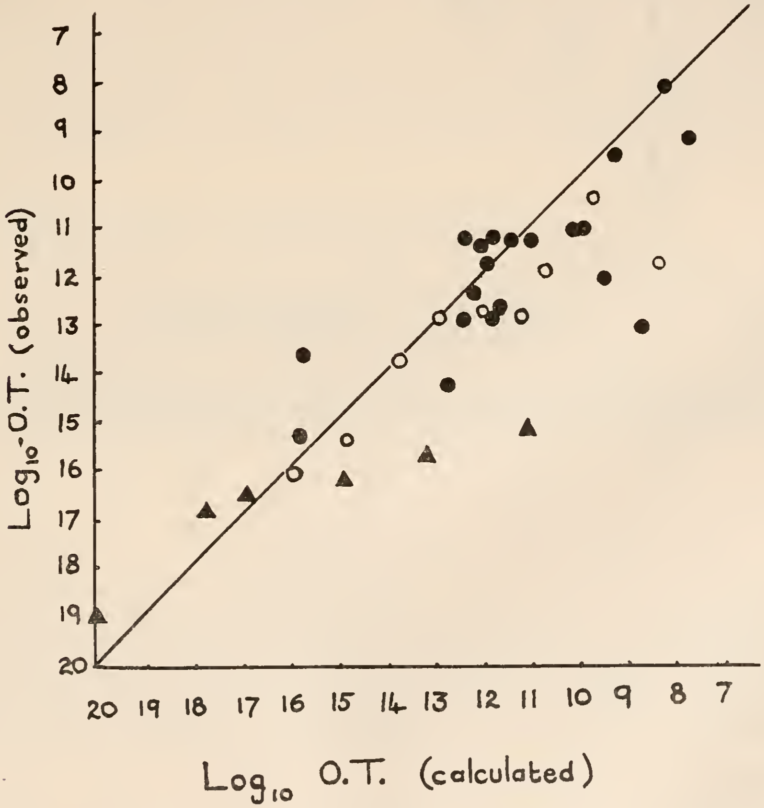

FIGURE 3. A comparison of observed olfactory thresholds and those calculated from equation (4) assuming that the molecules are spherical. ○ Values for normal alcohols; ▲ values for normal paraffins; ● other compounds.

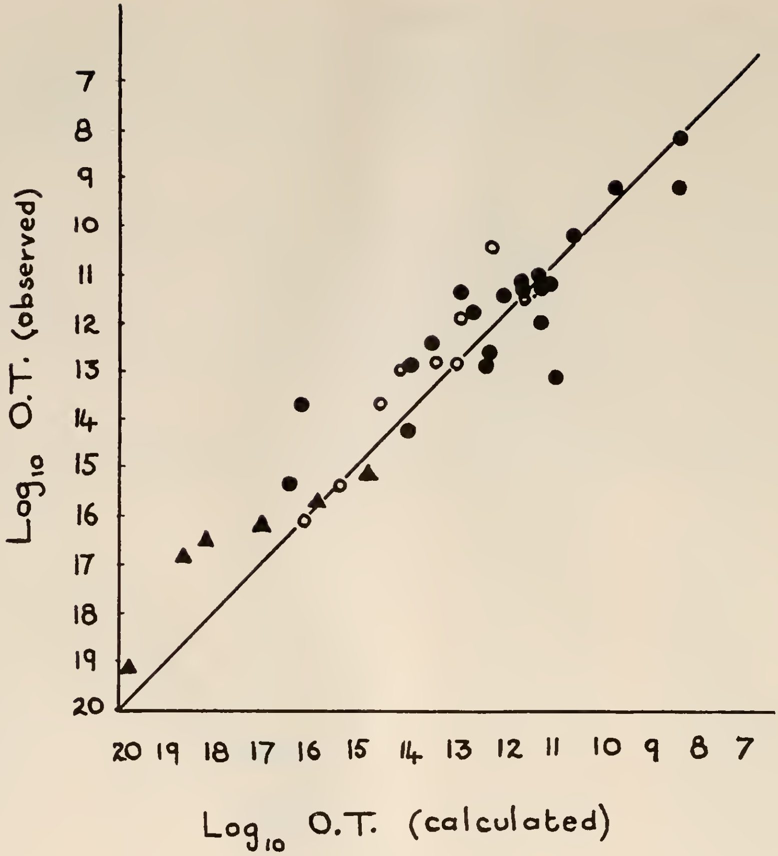

FIGURE 4. A comparison of observed olfactory thresholds and those calculated from equation (4) assuming that the odorant molecules are uncoiled and orientated in the olfactory cell membrane. ○ Values for normal alcohols; ▲ values for normal hydrocarbons; ● other compounds.

molecules are unfolded (Fig. 4) than with those obtained from the molecular volumes. The constancy of $1/p$ for the higher members of a homologous aliphatic series, as can be seen from equation (4), means that the thresholds for these compounds will decrease less rapidly than if $1/p$ increased continuously with chain length. This "tailing off" for the higher members of a series is observed for the thresholds of aliphatic alcohols, acids, hydrocarbons and with the chloroparaffins. The better agreement shown in Figure 4 (compared with Fig. 3) confirms the idea that the olfactory membrane is essentially fatty in nature.

(*b*) *Conditions at the olfactory cell surface*

In obtaining equation (4) from equation (3) by the use of experimental boundary conditions, values for log n (where n is the number of "sites" per cell) and α the area of one "site" are obtained. Log n is 4.64 which means that there are 4.5×10^4 "sites" per cell and α has the value of 64 Å² if the depth of the surface phase is taken to be 10 Å.

The active adsorbing surface area of each cell then comes to $n\alpha$ or 3×10^{-10} sq. cm. This is much smaller than that which can be calculated from the results of Bloom and Engström (1952) (3×10^{-6} sq. cm.) or those of Le Gros Clark and Warwick (1946) (ca. 10^{-8} sq. cm.). This could either indicate that the active "sites" do not occupy the entire cell surface or that one of the boundary conditions used in deriving (4) is wrong. If, for instance, there is a stronger odorant than β ionone (such that $\log \text{O.T.} < 8.2$), then the surface area predicted would be larger.

(*c*) *Prediction of olfactory thresholds*

If the values of $K_{O/W}$ and $K_{W/A}$ are determined experimentally for any substance, and if its molecular dimensions are known approximately, then it is possible by use of equation (4) to predict its olfactory threshold. Thus for cyclohexanol $\log K_{O/W}$ is 3.92, $\log K_{W/A}$ is 4.05 and from its molecular cross-sectional area, $1/p$ is 0.37 (using Figure 2). From equation (4) we now predict a threshold of 5×10^{11} molecules/cc.

Glycerol will have a value of about 0.32 for $1/p$ and the values of $\log K_{O/W}$ and $\log K_{W/A}$ are 1.93 and 4.0, respectively. The predicted threshold is then about 10^{14} molecules/cc. However, the saturation concentration in the air is much less than this (ca. 10^{13} molecules/cc.) at room temperature so that it cannot be detected.

(*d*) *The effect of temperature on the olfactory threshold*

The molecular dimensions of an odorant and therefore, presumably, $1/p$ will not alter as the temperature is raised and so the right hand side of (4) will be constant for any odorant. However, $K_{L/W}(K_{O/W})$ decreases for organic compounds as the temperature is raised; the value of $K_{W/A}$ also decreases numerically (thus at 20° C. $K_{W/A}$ for *n*-hexanol is 1.54×10^3 whilst at 40° C. it is 2.29×10^3).

For most compounds, therefore, $K_{O/A}$ decreases as the temperature is increased. This means that as the temperature is raised the olfactory threshold should attain higher values. This is in accord with the findings of Morimura (1934) who reported that small increases in temperature raise the threshold appreciably.

(*e*) *Olfaction and chemoreception in insects*

The receptors of olfaction in *Phormia* (the blowfly) are situated on the antennae and labellae whilst those of the contact chemical sense are on the tarsi. Dethier and his co-workers have been able to measure rejection concentrations for one sense by removal of the receptors of the other: thus by using antennectomized and labellectomized insects Dethier and Chadwick (1948, 1950) were able to measure rejection concentrations for tarsal chemoreception uncomplicated by olfaction. These concentrations are plotted in Figure 5 against $\log K_{O/W}$ and there is seen to be a linear relationship between the two quantities, the equation of the line being

$$\log M = 1.17 \log K_{O/W} + 2.83.$$

Here M is the threshold expressed as the molar concentration of test substance *in water* rejected by 50% of the flies. The slope of the line is very near to that expected for adsorption from solution on to a pure lipid membrane (1.0).

The olfactory rejection concentrations, however, (given by the molar concentrations in *the air* rejected by 50% of the insects) when plotted against log $K_{O/A}$,

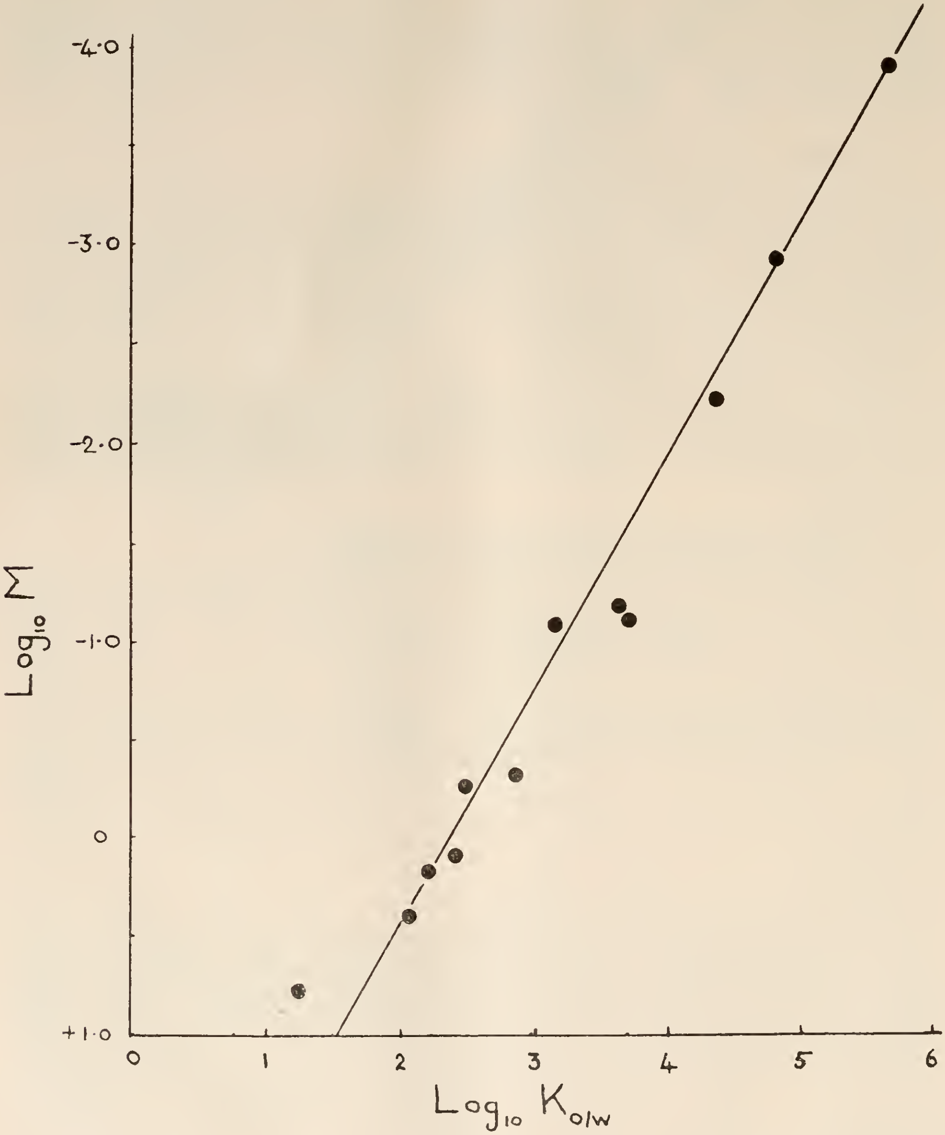

FIGURE 5. Plot of the logarithm of the molar concentration in water rejected by 50% of a number of blowflies against $\log_{10} K_{O/W}$. The concentrations are taken from the publications of Dethier and Chadwick (1948, 1950).

show a levelling off for the higher members of a homologous series (Fig. 6). This confirms that a shape factor in addition to an adsorption factor is significant in olfaction. Unfortunately, results are available only for aliphatic alcohols and aldehydes for *Phormia* (Dethier and Yost, 1952; Dethier, 1954).

Examination of the literature shows that there are many examples of the interaction of small molecules with biological membranes where adsorption is all-important (as with the chemoreception above or in haemolytic acceleration) and others where the effect is dependent on molecular shape or polarity in addition to the membrane concentration. Thus in the action of compounds on *B. typhosus* the effect is proportional to log $K_{O/W}$ (Ferguson, 1939) but the action of similar substances on chloroplast lecithinase systems (Kates, 1957) involves a shape or polarity factor in addition.

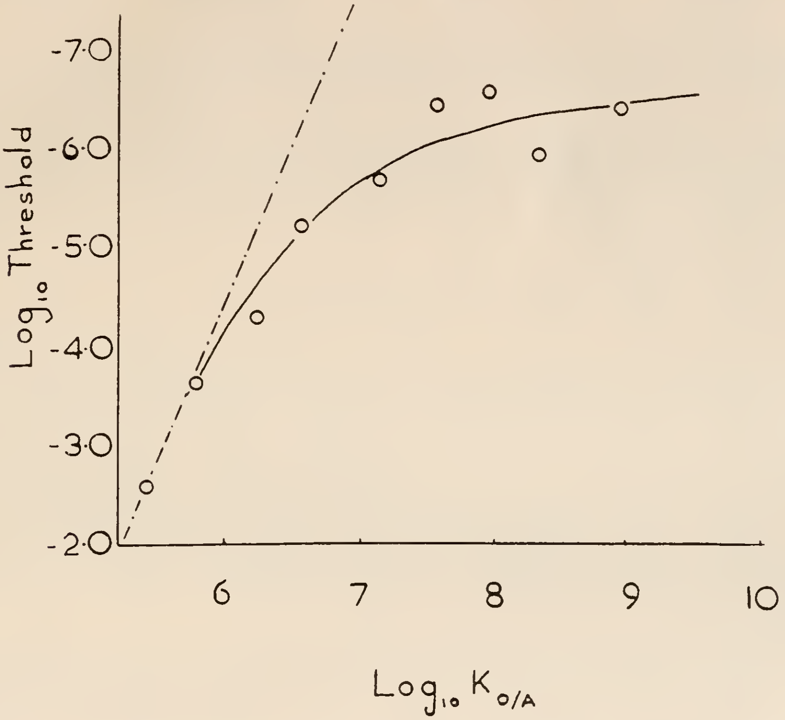

FIGURE 6. Plot of the logarithm of the olfactory rejection concentration for blowflies against $\log_{10} K_{O/W}$. The concentrations are taken from Dethier and Yost (1952). ·—·—·—·—., line with slope expected if $1/p$ were to increase regularly with chain length for alcohols.

(*f*) *Olfaction in dogs*

Several workers have recently determined olfactory thresholds for dogs (Krushinski, Chuvaev and Vollkind, 1946; Uchida, 1951; Neuhaus, 1953a, 1953b; Ashton, Eayrs and Molton, 1957). The carefully conducted experiments of Ashton, Eayrs and Molton, using solutions of fatty acids, yield thresholds in terms of gm. mols./liters of *aqueous* solution. From the values of $K_{W/A}$ given in Table I, these can be converted into molecules/cc. in air, assuming that equilibrium conditions prevailed during the experiments. The results for four acids are given below.

Thresholds

Acid	Log molar conc. in water	Molecules/cc. in air	
		Calculated	From Neuhaus (1953a, 1953b)
Butyric	-4.38	1.73×10^{10}	9×10^{3}
Valeric	-3.29	2.82×10^{12}	3.5×10^{4}
Caproic	-3.56	2.34×10^{12}	4.0×10^{4}
Oenanthic	-3.87	2.29×10^{12}	—

The thresholds are 10^7 times higher than those obtained more directly by Neuhaus and by Buytendijk (1921) and are of the same order as those observed for man. This means either that the experiments of Ashton, Eayrs and Molton were not carried out under equilibrium conditions or that dogs are less sensitive to odours than is commonly supposed. The levelling-off of the thresholds with the higher acids suggests, once more, that adsorption *and* molecular shape and size are important in olfaction.

(g) *Qualitative aspects of odour and the intensity factor*

The quantitative success of our theory supports, we believe, the idea that adsorbed odorant molecules initiate the nervous impulse by causing localized permeability changes in the cell membrane. It is clear that to be a strong odorant a substance must possess a large value of $K_{L/A}$ and a low value of p. Few molecules exhibit both characteristics since, if p is decreased by the introduction of branched chains, $K_{L/A}$ decreases markedly. Artificial musk (trinitro tertiary-butyl xylene) possesses good olfactory characteristics because the molecule is strongly adsorbed whilst $1/p$ is relatively high. Mullins (1955) has also concluded that rigid molecules are more effective stimulants than flexible molecules.

Mullins (1955) has recently shown that carefully purified *n*-paraffins definitely possess an odour and has measured thresholds for these compounds. The present theory, unlike those postulating interaction between polar groups, predicts this and the threshold values that we have calculated agree well with those observed by Mullins (Figs. 3 and 4).

It should be possible, by an extension of the theory, to investigate the variation in *intensity* of an odour with its concentration in the air about the threshold value. The intensity of an odour, however, is difficult to define or to measure for humans, and little work has been done on this subject. Experiments on *Phormia* or other insects seem more suitable for work on this parameter. The recent electrophysiological experiments of Beidler (1958) have shown that the activity of the olfactory receptors in the rabbit increases with the concentration of odorant until a maximum level of activity is reached.

It is not clear, however, whether excitation of an olfactory cell by an odorant is an all-or-nothing phenomenon (so that the intensity of smell depends on the number of cells stimulated), or whether there are different degrees of stimulation of one cell (when the intensity would depend on the number of impulses per second, *i.e.*, on the number of small areas per cell occupied by p odorant molecules).

Repeated exposure of the receptors to concentrations of odorant well above the threshold causes a temporary loss of sensitivity. On the theory of olfaction proposed here, this represents a simple depolarization phenomenon, the cell being unable to build up the "equilibrium" ion concentrations in the periods between successive inspirations. This phenomenon of fatigue or adaptation has been discussed by Adrian (1950a) with reference to olfaction in the rabbit and in general (1950b). Kristensen and Zilstorff-Pedersen (1953) discuss olfactory fatigue in man.

Two problems concerning the qualitative aspect of odour remain. Is there a relation between the adsorption and morphology of an odorant molecule and the type of odour it possesses? Secondly, is there more than one type of receptor in the olfactory epithelium?

The first point has been considered recently by Amoore (1952) and Timmermans (1954); the general conclusion is that molecular shape is important in determining the qualitative characteristics of an odorant. Amoore correlated the type of an odour with the shapes and electronic configurations of the molecules whilst Timmermans suggested that all molecules smelling like camphor are spherical, and conversely, that all spherical molecules have a camphorous smell. Unfortunately, there are exceptions to this rule which would otherwise be of the greatest significance.

In an exhaustive survey of work on the morphology of the olfactory region in vertebrates, Allison (1953) concludes that attempts (of this nature) to discover two or more types of olfactory receptor have been unsuccessful. Le Gros Clark (1957), however, has noted histological differences between receptor cells in different regions of the olfactory epithelium and has considered the possibility that this is linked with the problem of differential sensitivity.

Adrian (1950c) noted from his studies of the electrical activity of the olfactory bulb during stimulation that, since the receptors of lower animals are located in folds and are not equally accessible to currents of air, discharges set up by different smells may differ greatly in their temporal and spatial pattern. This fact alone is hardly sufficient to account for olfactory specificity since the olfactory epithelium in some higher animals, including man, is comparatively flat and exposed over its entire surface. His more recent electrophysiological investigations (1951, 1952, 1954) indicated that there are several types of olfactory receptor but that they are specifically sensitive at concentrations only just above the threshold; at concentrations much above this they react to more odorants. Beidler and Tucker (1955) recorded neutral activity from isolated bundles of primary olfactory nerves of the opossum. Their results, too, yielded evidence for a certain degree of specificity in the receptors. These techniques, however, because of the difficulties of placing the electrodes, yield indirect and imprecise information about different receptor types. We may conclude (with Allison) that any observed differences in receptor response must be attributed to subtle differences in structure at the physico-chemical level. Certainly, the specificities of the receptors might depend on the ease with which the different membrane layers are distorted: slight differences in their structure could render them more readily disoriented by molecules of certain shapes than by others. If there are only a few types of olfactory receptor, however, then finer shades of odour must be the result of a complex pattern of impulses arriving in the brain as a result of the different intensities of stimulation of different receptor types as suggested by Adrian. Mullins (1955) and Cheeseman and Mayne (1953) have studied this question with regard to olfaction in humans, by examining the interference of one odorant with the threshold of another. Mullins suggests that since butanol does not disturb the threshold for butane and vice versa, there are at least two types of receptor membrane in the olfactory epithelium.

The authors wish to express their gratitude to Sir Eric Rideal, F.R.S., for helpful advice during the course of this investigation.

SUMMARY

1. It is proposed that a stimulus is initiated in an olfactory receptor cell only when a critical number (p) of odorant molecules is concentrated within one small area of the cell membrane. An equation is derived which relates the olfactory threshold for humans to this number p and to the adsorption constant for molecules passing from air to the oil/water interface. Olfactory thresholds are calculated for a range of odorants on the assumption that $1/p$ is a function of the molecular cross-section area of an odorant. The calculated thresholds agree with the observed values; that predicted for glycerol exceeds the saturation concentration in the air so that this substance is odourless.

2. The equation suggests that olfactory thresholds should increase as the temperature is raised, as has been found experimentally. The results suggest that the olfactory cell membrane is lipoid in nature; the calculated "active surface area" of each olfactory cell is less than the observed total value. The effectiveness of compounds as odorants for *Phormia* and dogs, as well as for humans, depends on the concentration adsorbed on the membrane and upon the shape and size of the odorant molecules. Contact chemoreception in *Phormia*, however, is dependent only upon the appropriate adsorption constant and not upon molecular morphology.

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THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. II. CALCIUM DEPOSITION BY HERMATYPIC CORALS UNDER VARIOUS CONDITIONS IN THE REEF

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In a previous paper (Goreau, 1959a) a method was described for the direct determination of calcium deposition in madreporarian corals and other calcareous reef building organisms by the use of radioactive calcium-45. In these initial experiments calcification rates were measured under a variety of controlled conditions in the laboratory, but no information was obtained as to whether these were comparable with growth rates of corals in the open reef. Preliminary results suggested that growth in the natural environment is faster than that which we determined under laboratory conditions. In order to study this possibility more extensively we modified our method to allow direct assessment of calcium deposition in hermatypic corals under conditions approximating those found in the reef. For test organisms we selected fifteen of the most important West Indian shallow water hermatypic coelenterates, among which were included thirteen Madreporaria and two of the Milleporina.[2] These species were all collected from actively growing shallow reefs near Lime and Rackham Cays, located respectively two and four miles southeast of Port Royal, Jamaica, W. I. A general review of the abundance and distribution of these corals together with a description of Jamaican coral reefs has been published elsewhere (Goreau, 1959b).

PROCEDURE

All the experiments were conducted in the reefs mentioned above and the necessary equipment was brought to the site in a power launch which was moored in a suitable shallow place in about five or six feet for the duration of the operation. A boatman and two divers were required to carry out the various steps. Large twelve-liter Pyrex vacuum desiccator jars containing plastic-wrapped lead weights were used as the experimental vessels. These were sunk on the reef and selected coral colonies were gently detached by one of the divers who then transferred them into the jars in such a way that the living surface of the coenosarc was not

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[2] These species were *Acropora palmata* (Lamarck), *A. cervicornis* (Lamarck), *Porites porites* (Pallas), *P. furcata* (Lamarck), *P. astreoides* (Lamarck), *Siderastrea siderea* (Ellis and Solander), *S. radians* (Pallas), *Diploria clivosa* (Ellis and Solander), *D. strigosa* (Dana), *D. labyrinthiformis* (Linnaeus), *Colpophyllia natans* (Muller), *Manicina areolata* (Linnaeus), *Montastrea annularis* (Ellis and Solander), *Millepora alcicornis* (Linnaeus), *M. complanata* (Lamarck). The classification of the Madreporaria is according to Wells (1956), that of the Milleporina is according to Boschma (1948).

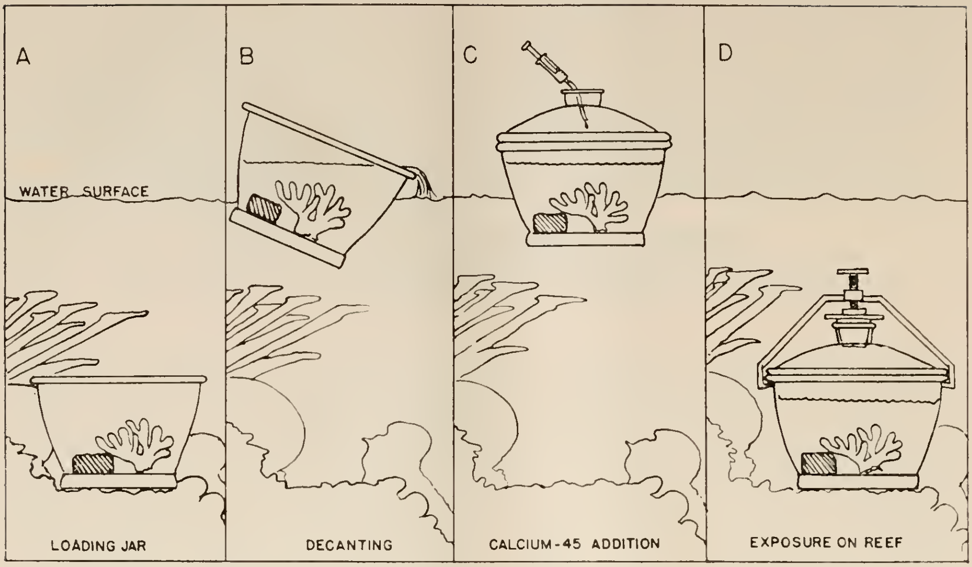

FIGURE 1. Procedure for loading and setting out the experimental jars on the reef. The cross-hatched square object on the bottom of the vessel is a plastic-wrapped lead brick used to weigh down the jar and stabilize it against currents and wave surge.

touched. The entire procedure was carried out under water and the coral colonies did not come in contact with air at any stage of the experiment. For replicate runs on the same species, colonies of similar size and shape were used. After loading, the jars were briefly raised above the surface, the excess water decanted, and ten milliliters of sea water containing approximately 0.2 mc. $Ca^{45} Cl_2$ adjusted to pH 8 were added. The lids were then put on, the jars gently swirled to mix the contents and a water sample taken for counting. The joint was sealed with silicone stopcock grease and the lids were secured by a bow clamp. The entire loading procedure is shown in Figure 1. Some of the jars were painted black to exclude light and one of these was run in each series. The full jars were placed by the divers on the reef at or near the sites from which the corals within had originally been taken, and situated so that there was no shading by neighbouring coral colonies. The standard depth was five feet, but several runs were also made at depths ranging from one to twelve feet. During our experiments the water temperatures were between 26° C. and 28° C. with fluctuations of not more than one degree centigrade during individual runs. Relative light intensities were estimated with a waterproof light meter. Although this instrument was not suitable for measurement of incident light, it could be used to give comparative light values by pointing it horizontally at a matte white surface about one foot distant.

The experiments were allowed to run for four to eight hours through the middle of the day from about 9:00 A.M. onward. They were terminated by picking up the jars from the reef, taking a water aliquot for counting, and transferring the corals to large volumes of fresh non-radioactive sea water with a pair of long tongs. The time of removal from the radioactive sea water was taken as the end point of the experiment. The dark jars were allowed to run for only about four hours, and opened first to minimize pH changes due to anaerobiosis.

The living colonies were rinsed for two hours in running fresh sea water to remove adhering radioactivity from the coenosarc. Ramose corals were sampled by the method described in our previous paper (Goreau, 1959a). Massive non-branching colonies were first split into smaller pieces with a cold chisel, cutting the samples from the surface in such a way as to include the whole thickness of the polypary layer.

A hollow steel core punch was used to obtain samples having a uniform cross-sectional area of 2 cm^2. With a hammer the punch was driven in several centimeters so that the entire thickness of the coenosarc was included. Care was taken to exclude from the sample any boring sponges, worms, clams, Crustacea and encrusting organisms. A shield and gloves were used to prevent contamination by radioactive coral fragments. Different parts of colonies were sampled to measure any growth gradients. The resultant cores were extruded by a piston, the method being illustrated in Figure 2. Cores from the deeper layers, just beneath the coenosarc, were taken to measure the diffusion of Ca-45 into the non-living parts of the skeleton. The coring method was not used on such corals as *Acropora cervicornis* and *Porites furcata* because of the relatively small size of their branches. These were therefore sampled by cutting or hack-sawing off pieces with accurately known dimensions from which the approximate surface areas could be calculated with suitable formulae. Measurements and sketches were made of all colonies treated in this way to locate the areas from which the samples had been taken. As these pieces were often large, some of them reaching several grams in weight, they were individually dissolved in numbered conical flasks containing 20 ml. dilute HCl (1 : 1).

After solution, all samples were heated, homogenized with a Potter tissue

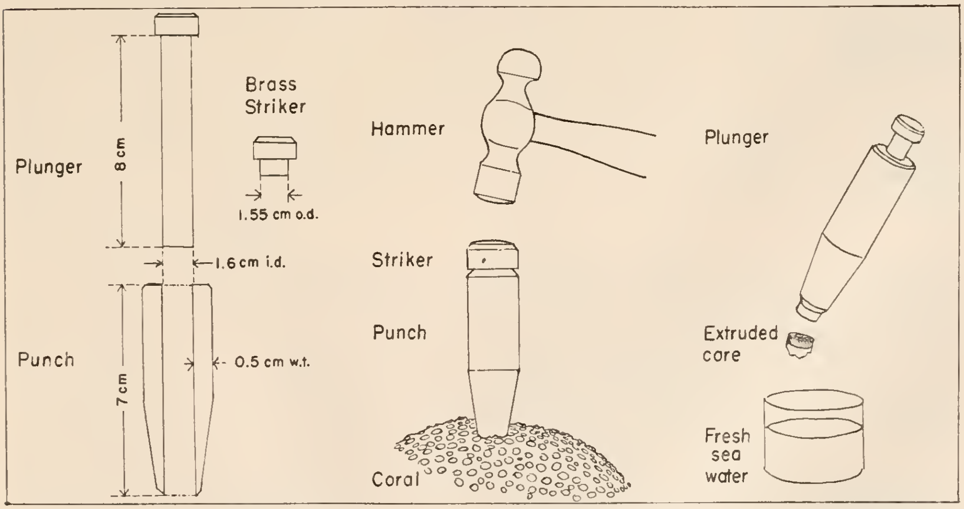

FIGURE 2. The core punch and the procedure for taking coral samples of known cross-sectional area.

Table I

Calcium deposition by hermatypic corals

Family and species	Branching or massive	Calcium uptake in µg. Ca/mg. N/hr. Sample numbers in parentheses			Maximum light:dark ratio
		Sunshine	Cloudy	Darkness	
Acroporidae					
A. palmata	B*	43.6 ± 9.81 (8)	26.3 ± 7.5 (10)	3.2 ± 0.64 (7)	13.5
		50.9 ± 8.40 (9)		4.0 ± 0.51 (8)	12.8
*A. cervicornis**	B*	53.7 ± 7.43 (9)	39.4 ± 8.65 (7)	8.4 ± 0.66 (6)	6.4
		71.5 ± 12.92 (5)	33.2 ± 6.80 (6)	4.1 ± 0.35 (8)	8.5
		61.0 ± 8.78 (6)			
		73.7 ± 18.82 (6)			
Poritidae					
*P. porites**	B*	25.1 ± 3.90 (9)		7.9 ± 4.60 (9)	3.2
		18.6 ± 3.31 (13)			
		23.2 ± 3.54 (8)			
		20.7 ± 1.73 (8)			
		29.3 ± 4.91 (8)			
		27.8 ± 5.58 (15)			
P. furcata	B*	15.4 ± 3.70 (8)		1.9 ± 0.20 (6)	8.1
		17.8 ± 4.25 (9)			
P. astreoides	M	6.7 ± 3.65 (8)		1.5 ± 0.36 (5)	4.5
		6.8 ± 1.35 (5)			
		13.7 ± 4.36 (8)			
		10.1 ± 2.20 (7)			
		5.5 ± 1.55 (7)			
		14.1 ± 3.00 (9)			
Siderastreidae					
S. siderea	M	13.7 ± 3.02 (11)			
		9.2 ± 2.50 (12)			
S. radians	M	7.1 ± 1.34 (6)			
Faviidae					
D. clivosa	M	6.7 ± 1.27 (8)			
		6.5 ± 1.00 (8)			
D. strigosa	M	6.5 ± 1.38 (11)			
		5.2 ± 1.11 (12)			
D. labyrinthiformis	M	20.9 ± 5.80 (15)			
		14.8 ± 2.72 (14)			
C. natans	M	14.9 ± 3.80 (11)			
		10.4 ± 1.85 (8)		2.5 ± 1.56 (8)	4.3
M. areolata	M				
zooxanthellae		13.4 ± 7.54 (10)		1.8 ± 0.42 (9)	7.5
no zooxanthellae		0.7 ± 0.31 (15)			

TABLE I—*Continued*

Family and species	Branching or massive	Calcium uptake in μg. Ca/mg. N/hr. Sample numbers in parentheses			Maximum light:dark ratio
		Sunshine	Cloudy	Darkness	
M. annularis	M	7.3 ± 1.71 (10) 8.9 ± 2.00 (10) 11.7 ± 2.00 (12)		0.3 ± 0.05 (9)	22.9
Milleporidae					
M. complanata	B*	35.7 ± 3.89 (9) 47.1 ± 13.80 (12) 36.2 ± 4.76 (6) 36.5 ± 3.92 (11)	24.6 ± 5.52 (9) 23.2 ± 4.43 (10)	4.7 ± 0.36 (10)	7.5
M. alcicornis	B*		23.6 ± 3.52 (8)		

* Apical polyps only.

grinder, cooled, and diluted to twenty-five milliliters with distilled water. Replicate aliquots were taken for counting and nitrogen analysis. The radioactivity was determined by the method described in our previous paper and the total amount of calcium taken up was calculated from the specific activity of calcium-45 in the sea water samples. The final calcification rate was expressed either in μg. Ca deposited per hour per mg. N, or μg. Ca deposited per cm^2. per hour. The final results were not corrected for isotopic exchange since the calcium-45 incorporated in this way amounted to a maximum of 0.5 per cent of the total calcium deposited.

RESULTS AND OBSERVATIONS

The highest calcification rates were invariably found in the terminal regions of the branching, or ramose corals, whereas lower rates were characteristic for the massive non-branching corals. Our results, in terms of calcium deposited per hour per milligram of organic nitrogen, are brought together in Table I. In decreasing order, the species we tested ranked approximately as follows: *A. cervicornis, A. palmata, M. complanata, M. alcicornis, P. porites, P. furcata, D. labyrinthiformis, C. natans, M. areolata, P. astreoides, A. siderea, M. annularis, S. radians, D. clivosa, D. strigosa.* The first six species are branching, the others are all massive non-branching.

As the ambient light intensity was previously shown to have an effect on the calcification rates of reef corals (*cf.* Goreau, 1959a), we tested the influence of different light conditions on coral calcium uptake in the reef by running some of our experiments on cloudy days, and some in complete darkness. Because exact measurements of the underwater light intensities could not be made due to lack of a suitable meter, we have listed our results in Table I under arbitrary headings, depending on whether the light conditions during the experiments were clear and sunny, cloudy or no light at all. In all species tested, the calcification rate was highest during sunny weather, lower during cloudy weather, and very low in darkness. The maximum light: dark ratios varied over a broad range, between approximately 3.2 to 22.9.

Previously we have also presented evidence that the calcification rate of corals is in part dependent on the presence of zooxanthellae, showing a marked fall in their absence (Goreau, 1959a). An opportunity came to repeat this experiment under natural conditions when several bleached and zooxanthella-less colonies of *M. areolata* were found alive and in good condition, growing unattached in semi-darkness under a large hollow coral head. The polyps of these were expanded, and their tissues were colourless and translucent. Controls were run at the same time with normal yellowish-brown colonies collected nearby. The results, which are included in Table I, show that the normal coral with zooxanthellae calcified about nineteen times faster than those which had lost their algae. This is in substantial agreement with our previous experiments. The light : dark ratio for normal colonies of *M. areolata* was about 7.5, which means that colonies with zooxanthellae could still calcify considerably faster in darkness than could zooxanthella-less colonies in the light. An hypothesis concerning a mechanism whereby the calcification process in Madreporaria may be stimulated by photosynthesizing zooxanthellae has been published in the paper cited above.

Table II

Calcification rates in different parts of branches of A. cervicornis at various light intensities

Light conditions on the reef	Calcium uptake in µg. Ca/mg. N/hr. Sample numbers in parentheses		
	Apical polyps	2 cm. behind apex	3 cm. behind apex
Darkness	8.4 ± 0.66 (6)	2.2 ± 0.72 (6)	
Cloudy weather	39.4 ± 8.65 (7)	15.1 ± 6.20 (8)*	
	33.2 ± 6.80 (6)	9.8 ± 3.91 (5)*	
Bright sunshine	53.3 ± 7.43 (9)	33.9 ± 3.47 (10)	27.5 ± 4.45 (7)
	71.5 ± 12.92 (5)	48.8 ± 8.39 (4)	28.9 ± 1.00 (5)
	61.0 ± 8.78 (6)	42.5 ± 7.15 (7)	31.6 ± 5.39 (7)
	73.7 ± 18.82 (6)	36.8 ± 6.57 (5)	25.7 ± 3.61 (6)

* Samples taken 3 cm. behind apical polyp.

Considerable variance in the calcification rates is evident in our results, both within sample groups taken from the same colony, as shown by the standard deviation of the means, and between different colonies of the same species. To determine the probability that these two forms of variance were due to dissimilar causes, the data from four species[3] were analysed by the F test (*cf.* Snedecor, 1946; pp. 232–233). This shows that the F values varied from less than one to less than five per cent, thus indicating that differences in calcium uptake rate between uniformly sized colonies of the same species growing under similar conditions were significant. Similar variances almost certainly occur in most of the other species tested.

Preliminary tests to determine the effect of depth were carried out on three coral species simultaneously at 1, 3, 6, 9, and 12 feet. The results were inconclusive, showing no correlation of the calcification rate with depth over the range

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[3] *A. cervicornis, P. porites, P. astreoides, M. complanata.*

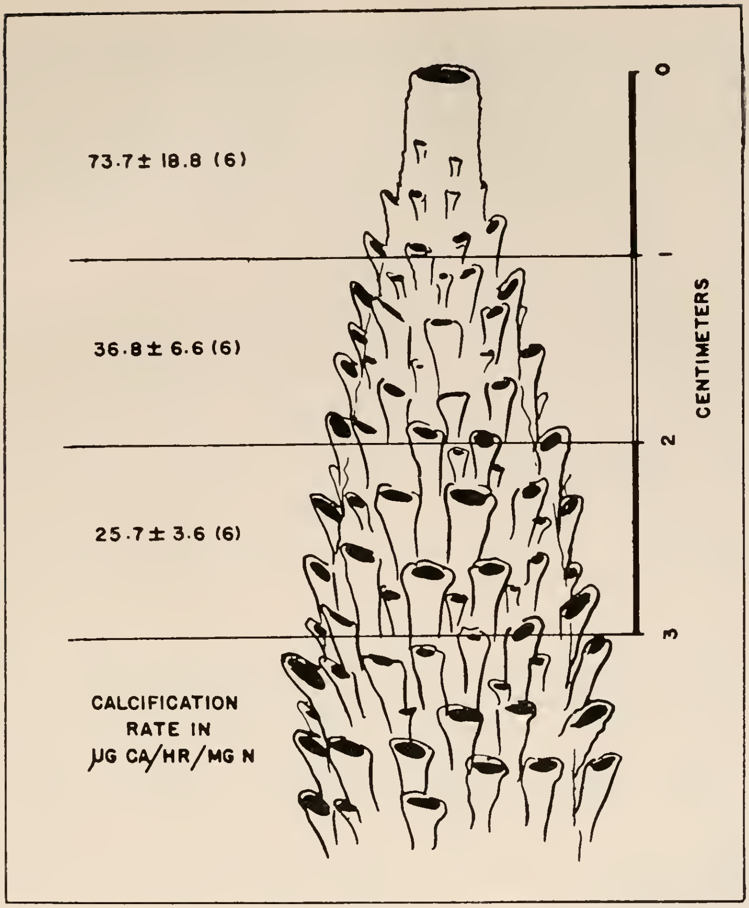

FIGURE 3. Apical region of a branch of *Acropora cervicornis* showing the existence of a calcification gradient. The large terminal polyp at the top has the highest rate of calcium incorporation.

tested probably because the light intensity was more or less uniform due to backscatter by sandy patches in that part of the reef where these experiments were made. Further work correlated with more precise measurements of the incident light intensity over a greater depth range is now in progress.

Calcification rates in the ramose corals tended to decrease systematically from a maximum in the apical polyps to much lower rates in the lateral and basal branch corallites. A characteristic gradient is shown in Table II and Figure 3 for the staghorn coral, *A. cervicornis*, where the calcification was measured at one-centimeter intervals away from the branch apex. Systematic variations in the calcification rates were also observed in other branching species, *e.g.*, *A. palmata*, *M. complanata* and *P. furcata* as shown in Table III. In the massive corals tested, gradients were absent and such variations as were observed appeared to be random over the entire colony.

Some preliminary experiments were also carried out to determine the calcification rate in terms of surface area.This was done by measuring the calcium uptake in core samples of known diameter and cross-sectional area. Our results are almost certainly too high, due to an uncompensated systematic error introduced by the difficulty of measuring the total area of the complicated skeletal surface of corals. Pending development of an accurate method for doing this, a problem now under investigation in our laboratory, the area of our samples has been calculated from their geometry based on smoothed out dimensions, and our results can therefore only be interpreted in very approximate terms on the assumption that all errors in the method are predominantly in the same direction. In column 1 of Table III are shown the approximate calcium uptakes per cm.^{2} in four ramose and two massive corals. It is interesting to note that the calcification rate per unit area of the two non-branching corals seems to be equal to or greater than that of the branching species. The reverse is true when calcium uptake is estimated in terms of the nitrogen content as shown in column 3 of Table III. In the second column of this table are listed the approximate tissue biomasses contained in the sample in terms of mg. N/cm^2. This shows that the two massive faviid corals had a higher content of organic matter per unit area than the branching acroporid, poritid, and hydrocoralline species that were tested. This confirms what is readily seen by naked eye, that the acroporid and hydrocoralline corals have a very thin translucent

Table III

The relationship of approximate geometric area to protein nitrogen content and calcium uptake in corals. Sample number in parentheses

Species and location of samples	Branching or massive form	Approximate μg. Ca/hr./cm.^{2}	Approximate mg. N/cm.^{2}	μg. Ca/mg. N/hr.
A. palmata	B			
Outer edge of frond		69 ± 9.5 (3)	2.4 ± 0.4 (3)	29.5 ± 0.78 (3)
7 cm. behind edge, upper		52 ± 2.0 (3)	3.0 ± 0.3 (3)	17.8 ± 2.59 (3)
7 cm. behind edge, under		32 ± 4.0 (3)	1.8 ± 0.7 (3)	17.5 ± 0.05 (3)
A. cervicornis	B			
Apical polyps		20 ± 1.7 (9)	0.5 ± 0.05 (9)	41.7 ± 11.7 (9)
3 cm. behind apex		12 ± 1.8 (6)	0.6 ± 0.01 (6)	17.4 ± 2.95 (6)
5–7 cm. behind apex		10 ± 1.6 (3)	0.8 ± 0.4 (3)	13.8 ± 0.84 (3)
M. complanata	B			
Superior edge of frond		38 ± 5.7 (9)	1.1 ± 0.1 (9)	35.7 ± 3.89 (9)
10 cm. behind edge		12 ± 1.8 (4)	2.0 ± 0.3 (4)	5.6 ± 0.22 (4)
P. furcata	B			
Apical 1 cm. of branch		36 ± 6.7 (8)	2.4 ± 0.1 (8)	15.4 ± 3.70 (8)
2 cm. behind apex		6 ± 1.4 (3)	3.3 ± 0.9 (3)	3.34 ± 0.94 (3)
5 cm. behind apex		4 ± 2.9 (2)	3.1 ± 0.6 (2)	3.07 ± 0.55 (2)
C. natans	M			
Random over whole colony		80 ± 1.2 (8)	7.9 ± 1.2 (8)	10.4 ± 1.85 (8)
M. annularis	M	54 ± 11.9 (3)	7.3 ± 0.6 (5)	7.32 ± 1.71 (5)

coenosarc; the poritids a somewhat thicker, more opaque one; and that the faviids on the whole are rather fleshy, especially the species analysed here. The possibility thus arises that the calcification powers of the massive corals, which are less marked than those of the branching species on the basis of tissue mass as expressed by the nitrogen content, may be as great or greater per unit surface area of the basal calicoblastic epidermis which is the organ of skeletogenesis in corals. The problem is now under more detailed investigation.

Discussion

The long-term field observations of Wood-Jones (1910), Mayor (1924), Edmondson (1929) and the Stephensons (1933) directed attention to the fact that corals do not necessarily grow at even rates. The experiments of the Stephensons were particularly valuable as they established that individual variations among healthy and normal corals of the same species growing for the same length of time in the same environment gave very irregular results, leading to the conclusion that the use of averages was preferable to individual figures. Our data seem to corroborate this view to the extent of showing that there are significant variations in calcification rates among similar colonies of the same species under identical environmental conditions over the comparatively short periods of time during which our measurements were made. It is, however, uncertain whether the variances we observed in experiments lasting only a few hours are similar to those described in the studies of other investigators, all of which were made under non-uniform conditions over periods of months to years. It should be understood that because of the dissimilarity in aims, methods and expression of results, the calcification rates cited in this paper cannot be compared with the coral growth data obtained by previous observers who used completely different procedures. We have endeavoured to determine calcium uptake of corals and to use this as an index of physiological function, not as a direct measure of absolute growth rate. We hope nevertheless that the hiatus between these two objectives will disappear with further work.

The present results also support our earlier observations (Goreau, 1959a) that the calcification rates of individual corallites fluctuate widely, and probably at random, even in symmetrical colonies. Statistically, this type of variance appears to be separate and distinct from the clonal growth differences that occur among individual colonies in a population of a given coral species.

The local fluctuations in the calcium uptake of individual corallites may be related to the manner in which the coral skeleton is laid down and organised. The sclerenchyme, or skeleton, consists of a succession of discontinuous layers held together by walls and partitions: the dissepiments, thecae and sclerosepta, respectively. The dissepiments are separated from each other by more or less regular horizontal spacings which suggests that the process of deposition is itself discontinuous, possibly involving local and temporary detachment of the calicoblast from the skeletal basis just before the secretion of a new layer, a millimeter or so above the old. Circumstantial evidence that the attachment of the polyp to its skeleton may be under some degree of facultative control is provided by the observation that polyps of starving corals are able to detach themselves completely from the corallum. In this phase they can stay alive for some weeks without showing any evidence of renewed skeletogenesis although they are able to ingest food normally (Goreau, unpublished observations). We do not suggest that the coenosarc ever becomes entirely detached under normal circumstances, but the fact that it is able to do so at all indicates that the polyps are not anchored down quite as securely as is at present believed. On the basis of mineralogical evidence, Bryan and Hill (1941) have also suggested that madreporarian calcification is a discontinuous process involving possible temporary detachment of parts of the polyp.

TABLE IV

Comparison of calcification rates of some hermatypic corals with their relative abundance on a large Jamaican coral reef

Family and species	Relative abundance in all zones of reef	Relative calcification rate
Acroporidae		
A. cervicornis	+++	oooooo
A. palmata	++++	ooooo
Poritidae		
P. porites	++	oooo
P. furcata	+++	oo
P. astreoides	++++	o
Siderastreidae		
S. siderea	++	oo
*S. radians**	++	o
Faviidae		
D. clivosa	++	o
D. strigosa	+++	o
D. labyrinthiformis	+	ooo
C. natans	++	oo
*M. areolata**	+	oo
M. annularis	++++	oo
Milleporidae		
M. complanata	+++	ooooo
M. alcicornis	+ (?)	ooo

Legend:	+	not common	o	0– 9 μg. Ca/mg. N/hr.
	++	common	oo	10–19 μg. Ca/mg. N/hr.
	+++	abundant	ooo	20–29 μg. Ca/mg. N/hr.
	++++	very abundant and dominant in some zones	oooo	30–39 μg. Ca/mg. N/hr.
			ooooo	40–49 μg. Ca/mg. N/hr.
			oooooo	50+ μg. Ca/mg. N/hr.

* Colonies numerous but of small size with low aggregate biomass.

We have so far found no consistent relationship between calcification rates of corals in terms of organic matter, and their prevalence on a coral reef. In Table IV the relative calcification rates of the species tested are compared with the relative abundance of the same corals on a large Jamaican reef. For the Acroporidae and Milleporidae, which are the predominant hermatypes of the shallowest reef zones, there may be some correlation of abundance with calcium uptake. In the Siderastreidae, the much larger and more common *S. siderea* appears to calcify faster than the smaller but hardier *S. radians*. However, in the Poritidae, *P. astreoides* is the more frequent and important species in Jamaican reefs, although it calcifies much less rapidly than its branching congeners *P. porites* and *P. furcata*. In the Faviidae, the apparent discrepancy between the occurrence on the reef and calcification rate is still more pronounced since the faster calcifying species such as *C. natans, D. labyrinthiformis* and *M. areolata* are less prevalent on the reef than the slower growing *D. strigosa, D. clivosa* and *M. annularis*.

Although the preeminence of the massive corals may in part be due to the greater susceptibility of branching corals to damage by heavy seas, this fails to explain why branching corals are in general typical of the turbulent shallow waters whereas the massive corals are more characteristic of the deeper calmer regions of the reef. Despite the fact that the growth rate of the massive coral *M. annularis* (*cf.* also Vaughan, 1915, and Vaughan and Wells, 1943, p. 64) is relatively low, in Jamaica this species is nevertheless one of the most important hermatypes in regard to the total quantity of calcareous material contributed to the framework of the reef. It is usually the dominant coral in the deeper regions of the fore-reef, reaching a peak of development in the buttress zone where the gigantic size of its colonies is evidence of extremely vigorous proliferation (Goreau, 1958a, 1959b). This zone is named for the great coral promontories which grow upward and forward into deeper water, forming immense dentate projections that are oriented with their long axis into the prevailing seas so that they present a maximal area for coral growth with a minimal frontal area exposed to the surge of the waves. In the buttress zone the growth habit of such massive corals as *M. annularis* and *P. astreoides* becomes curiously flattened so that the colonies often resemble platters, roof shingles or great flow sheets, whereas in other shallower zones in which these corals are frequently much more exposed to surf and currents their colonial habit is mushroom shaped. If it is assumed on the basis of our preliminary data that the growing power of reef corals is proportional to the area of the calicoblast, then the formation of buttresses and a flattened growth habit may be interpreted as representing adaptive modifications which enable massive corals to compete more successfully on an area basis with the faster growing ramose corals.

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Summary and Conclusions

1. The calcium uptakes of 13 hermatypic corals and 2 hydrocorallines were measured by a modified calcium-45 method under conditions approximating those of the natural environment of the reef in experiments lasting four to eight hours.

2. When measured on the basis of nitrogen content, the growth rates of the branching corals were higher than those of the massive corals. On the basis of area, however, the latter appear to grow as fast or faster than the former.

3. Light intensity had a profound influence on the growth rate under the conditions of our experiments. All corals tested deposited calcium fastest in sunlight, less during cloudy weather and least in darkness. Bleached zooxanthella-less colonies deposited calcium at lower rates in the light than normal colonies with zooxanthellae did in darkness.

4. Systematic calcification gradients were observed in branching corals but not in massive species.

5. Although there was a considerable variance in the growth rate from place to place within individual colonies, we also observed large and significant differences in the growth rate between individual colonies of the same species, size and shape under similar conditions.

6. It is suggested that the organisation of the skeleton, which is really composed of many separate lamellae with spaces in between, indicates that the calcification process itself may be discontinuous, and that this may in turn be responsible for the growth fluctuations that were observed within the coral polyp populations of individual colonies.

7. No hard and fast correlation was observed between the calcification rates per unit of nitrogen and the relative abundance of the species on the reef. Although some of the commonest shallow water corals are very fast calcifiers as well, the most important West Indian reef builders are the slower growing massive corals.

8. An explanation is put forward to the effect that growth of massive corals in the reef is enhanced by the formation of buttresses which serve to increase the available surface area for calcification.

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DISTRIBUTION OF A RADIOMERCURY-LABELLED DIURETIC (CHLORMERODRIN) IN TISSUES OF MARINE FISH[1]

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The ability of mercurial compounds to increase urine flow, recognized at least since the time of Paracelsus (1493–1541), has been widely applied in clinical medicine following the introduction of the organic mercurial diuretics (Saxl and Heilig, 1920). The association of suppression of urine flow with poisoning due to inorganic mercury compounds (Woodman and Tidy, 1877) also suggests that renal tissue is particularly susceptible to the action of such substances. Ludwig and Zillner (1890) were among the early investigators who demonstrated by chemical analysis that the kidneys of dogs poisoned with mercuric chloride contain far higher concentrations of mercury than other tissues. Many subsequent studies, with the use of both biochemical and radioisotopic methods, have confirmed this finding.

Hg^{203}-labelled chlormerodrin has recently been used to determine more precisely the location of mercury deposition in mammalian kidney. In dogs and in rats the concentration is highest in the outer renal cortex, decreases in the medulla, and is lowest in renal papillary tissue (Greif *et al.*, 1956). In this connection, Walker and Oliver (1941) have pointed out that the rat outer renal cortex is composed largely of proximal segment of the tubule. The purpose of the present study was to determine, with the use of a radiomercury-labeled organic mercurial diuretic, whether mercury is selectively concentrated in the kidneys of marine fish with a variety of nephron types.

MATERIALS AND METHODS

Specimens of flounder (*Paralycthys dentatus*), toadfish (*Opsanus tau*) and dogfish (*Mustelus canis*) were furnished by the Collecting Department of the Marine Biological Laboratory, and were stored briefly in live cars before use. They were weighed and injected with alkaline solutions of chlormerodrin[2] in concentrations indicated in the tables. Most injections were made intramuscularly in the fleshy part of the tail, although a few intramuscular injections were placed immediately dorsal and posterior to the gills. The fish were placed in a running sea water aquarium until sacrificed. At the time of killing, the fish were stunned and bled from either the tail vein or the heart with a needle attached to a heparinized syringe. Tissue was then rapidly removed from the fish, weighed on a torsion balance, and transferred to a test tube for counting in a well-type scintillation counter with scaler. Blocks of tissue were removed from the anterior, medial, and

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[2] Generously provided by Dr. R. F. Pitts.

posterior portions of the kidney for counting. Results are expressed as milligrams chlormerodrin mercury per gram wet weight of tissue. Unless otherwise indicated, the middle portion of the kidney was selected for comparison with other excised tissues. Urine was obtained either by inserting a pipette into the urogenital papilla or aspirating the exposed bladder with a syringe and needle. Phenol red accumulation in the excised flounder tubule was studied by the method of Forster and Taggart (1950).

RESULTS

a) *Flounder.* Under the present experimental conditions a high tissue mercury concentration is always found in the kidneys. This is especially striking at the dosage level of one milligram chlormerodrin mercury per kilogram body weight (Table I), an amount which will produce diuresis in mammals (Borghgraef *et al.*, 1956). The larger dose, 10 milligrams mercury per kilogram (Table II), which is toxic to mammals, also proved fatal to some of the fish. Kidney tissue excised from flounder surviving the larger dose was unusually friable. On microscopic examination, the freshly teased tubules appeared cloudy and granular and failed to concentrate phenol red. When such tissue is fixed in Susa fluid and stained with hematoxylin and eosin it cannot be readily distinguished from kidney of fish given

TABLE I

Mean tissue concentration of radiomercury administered as one milligram labelled chlormerodrin mercury per kilo body weight. All values expressed as micrograms mercury/gram wet weight of tissue

Species	No. of Animals	Plasma	Kidney	Liver	Bile	Gills	Heart	Stomach	Gonads	Spleen
12 Hours After Intramuscular Injection										
Flounder	2	0.7	52.2	3.2	9.7	0.2	0.5	0.4	0.2	0.7
Toadfish	1	0.8	13.2	0.3	0.0	0.5	0.0	0.5	0.0	0.2
Dogfish	1	0.9	42.2	4.4	21.9	0.0	0.0	0.3	0.8	0.5
24 Hours After Intramuscular Injection										
Flounder	3	0.3	80.1	3.9	1.7	0.2	0.4	0.2	0.2	0.5
Toadfish	2	0.7	12.0	0.3	0.2	0.5	0.5	0.4	0.1	1.0
Dogfish	3	0.6	14.5	4.0	33.6	0.4	0.2	2.0	0.9	0.4
48 Hours After Intramuscular Injection										
Flounder	2	3.5	35.6	6.7	2.8	1.9	0.2	0.4	0.3	1.0
Toadfish	2	0.3	9.0	0.4	0.2	0.3	0.3	0.2	1.2	0.4
Dogfish	1	0.2	6.4	2.7	21.0	0.9	0.3	2.9	0.7	0.4
72 Hours After Intramuscular Injection										
Flounder	2	0.1	24.6	5.7	2.7	0.2	0.2	0.0	0.2	0.7
Dogfish	1	0.1	4.4	7.3	114.0	1.0	0.2	0.4	0.7	0.4

TABLE II

Mean tissue concentration of radiomercury after administration of 10 milligrams labelled chlormerodrin mercury per kilo body weight. All values expressed as micrograms mercury/gram wet weight of tissue

Species	No. of Animals	Plasma	Kidney	Liver	Bile	Gills	Heart	Stomach	Gonads	Spleen
12 Hours After Intramuscular Injection										
Flounder	4	13.6	288.0	55.6	12.8	12.5	8.2	8.7	7.3	30.2
Toadfish	2	29.5	197.0	15.0	2.6	12.3	23.5	11.7	6.4	16.5
24 Hours After Intramuscular Injection										
Flounder	4	11.9	146.5	51.8	53.7	11.4	9.3	6.0	6.4	30.1
Toadfish	3	19.7	128.9	29.2	20.0	10.4	21.2	12.0	8.4	13.6
48 Hours After Intramuscular Injection										
Flounder	3	12.6	77.1	59.2	121.0	10.3	8.6	8.5	9.8	37.5
Toadfish	3	19.0	105.0	36.2	37.8	11.0	21.4	10.2	5.0	16.6
72 Hours After Intramuscular Injection										
Flounder	2	10.2	89.4	45.1	57.0	7.1	4.2	7.7	6.7	19.6
Toadfish	3	20.8	106.5	40.9	55.7	11.7	29.2	11.5	18.5	15.8

the smaller dose of mercurial or from that of control flounder, although these latter tissues concentrated phenol red and appeared normal when viewed with the dissecting microscope. On the regimen of 10 milligrams Hg per kilogram, high concentrations of mercury are seen not only in the kidney but also in the liver and bile of the surviving animals.

b) *Toadfish.* In Table I and Table II may be found the distribution of chlormerodrin mercury in the tissues of this species. As in the flounder the mercury concentration in the kidney is high, but at 10 milligrams mercury per kilogram the concentration in the bile in toadfish does not exceed the concentration in the kidney. It was possible to obtain blood and urine samples in 11 toadfish after chlormerodrin injection. Despite great individual variation, in all but one animal the urine-to-plasma ratio was at least 4, and the mean value for this ratio was 30.7 ± 9.6 (S. E.). Phenol red, injected with the chlormerodrin, also appeared in the urine.

c) *Dogfish.* Tissue mercury distribution in a small number of animals is included in Table I. The most striking difference to be seen in this instance is the high mercury concentration in the bile on the one milligram mercury per kilogram body weight regimen.

General. In all animals at the one milligram/kilogram dose the kidney mercury concentration is high, and only in the dogfish is the concentration in the liver and in the bile impressive. It is notable that in none of the animals on any regimen is the mercury concentration in either the gills or the stomach higher than in the

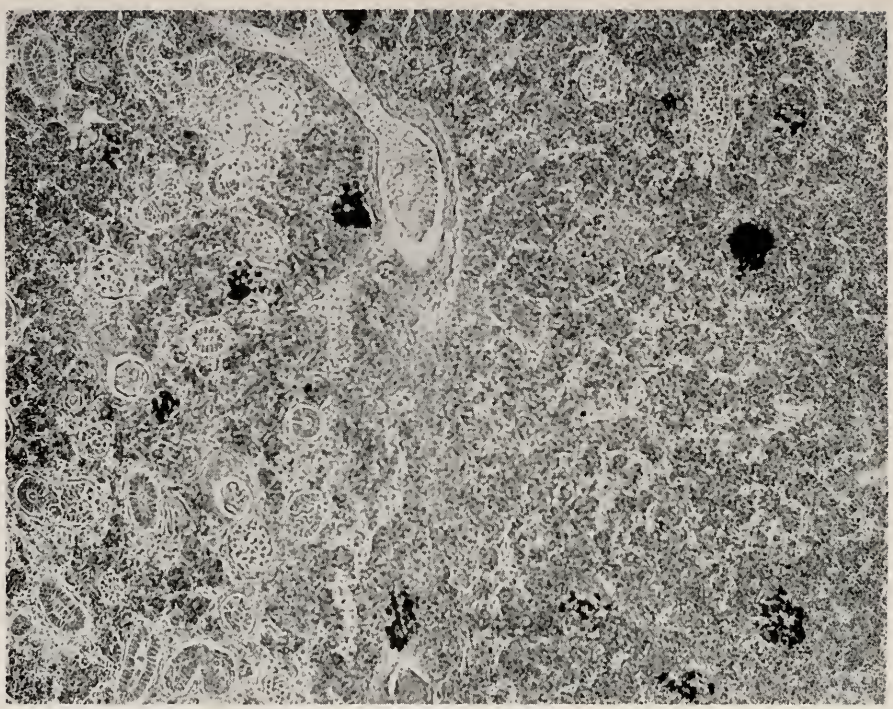

FIGURE 1. Photomicrograph of area of flounder kidney showing transition between anterior portion (right) containing few renal elements, and middle portion in which numerous glomeruli and tubules can be seen.

plasma. The regional distribution of mercury in the kidney is of interest. Both flounder and toadfish kidneys contain much lymphoid tissue in which the renal elements are imbedded, whereas the dogfish kidney on microscopic section shows tubular and glomerular structures surrounded by supporting tissue containing few or no lymphoid elements. Figure 1 shows a photomicrograph of the transition zone

TABLE III

Regional distribution of radiomercury in fish kidney. Combined data from fish injected with one milligram and 10 milligrams chlormerodrin mercury per kilogram and studied at time intervals up to 72 hours after injection. Results expressed as per cent of mean mercury concentration of tissue from the three regions of the kidney sampled

Species	No. of animals	Per cent of total mean mercury concentration in the kidney contributed by:		
		Anterior sample	Middle sample	Posterior sample
Flounder	16	9.0 ± 8.5*	48.5 ± 5.9	42.5 ± 5.6
Toadfish	15	26.6 ± 3.6	33.9 ± 5.1	39.6 ± 6.5
Dogfish	6	24.9 ± 7.6	35.4 ± 3.7	39.9 ± 8.2

* Standard deviation.

between the anterior and middle section of the flounder kidney. A few renal elements can be seen imbedded in the lymphoid cells in the anterior portion, and these elements can be seen in larger numbers in the more posterior portion of the section. The mercury concentration of the anterior block from which this particular section was made is one fifth that found in the more posterior section.

Table III represents a summary of the regional distribution of radiomercury in the kidneys of the species studied. The mean mercury concentration in anterior, medial, and posterior samples is assigned the value of 100%, and the proportion of the total contributed by the three regions is also expressed as per cent. It can be seen that in each species the lowest mercury concentration is found in the anterior portion, but that in the case of the flounder the difference is particularly striking.

DISCUSSION

The species of marine vertebrates chosen for this study have nephrons which can be classified roughly into three types. The flounder can be considered to have kidneys with glomerular and proximal segmental function (Forster and Hong, 1958); the dogfish has a complex nephron, with glomerulus, proximal, and distal segment (Kempton, 1943). The toadfish, since the original description by Marshall (1929), has been considered to be both functionally and anatomically an aglomerular species with a nephron consisting of a proximal segment only.

The present experiments show clearly that the presence of a proximal segment is sufficient for a nephron to be capable of concentrating mercury. The high U/P ratio for mercury observed in the toadfish agrees with the findings in dogs of Borghgraef *et al.* (1956) that chlormerodrin mercury is excreted by means of a secretory mechanism. Subsequent "stop-flow" experiments have further localized this mechanism in the dog proximal tubular segment (Kessler *et al.,* 1958). Giebisch and Dorman (1958) have noted the selective accumulation of radiomercury administered as labeled chlormerodrin in the kidneys of carp, chicken, bullfrogs, and turtles, and have shown that in *Necturus maculosus,* a species in which the proximal and distal tubular segments are anatomically distinct, the proximal segment always contains a higher mercury concentration than does the distal. The observation of Bieter (1933) that mercuric chloride induces a diuresis in the anaesthetized toadfish also suggests that mercurial compounds act proximally. Unfortunately no analyses of toadfish urine for sodium and chloride are given with Bieter's experiments. The excretion of phenol red by the toadfish in the present experiments was also observed by Shannon (1938) and others.

The regional distribution of radiomercury noted in Table III deserves some comment. The injections of labelled chlormerodrin were made, for the most part, into the muscular portion of the tail, and since the kidneys of fishes are supplied largely by the renal portal circulation (Smith, 1953), it might be expected that the portion of the kidney closest to the injection site would contain the highest concentration of radiomercury. Such an explanation would agree with the results in both toadfish and dogfish, as seen in Table III, but in the case of the flounder the highest concentration of radioactivity is found in the medial portion of the kidney. It seems probable, therefore, that the low concentration of radiomercury in the anterior portion of the kidney in this species is a reflection of the small number of renal elements imbedded in the lymphoid tissue in this region (Fig. 1) and that this represents an example of the strikingly higher accumulation of mercury by renal elements than by lymphoid tissue.

Mercurial diuresis in mammals is associated with an increased excretion of sodium and chloride ions. In the case of the marine fish, excretion of the sodium and chloride ions taken in via the mouth appears to be accomplished by the gills (Black, 1957). It is therefore interesting to note that these structures do not accumulate radiomercury under the present experimental conditions. Certain compounds of mercury are concentrated by the mammalian kidney to the same degree as is chlormerodrin mercury without affecting the excretion of water, sodium, or chloride (Kessler *et al.*, 1957). The present experiments with marine fish furnish evidence that the accumulation of mercury against a concentration gradient is a process which may occur in tissues not usually involved in sodium and chloride excretion. Studies in progress with the nephridia of *Phascolosoma gouldi* (Greif, 1957) are in agreement with this evidence.

SUMMARY

1. Chlormerodrin labelled with radiomercury has been injected into marine fish with different types of nephrons. The kidneys of flounder (*Paralycthys dentatus*), with glomerulus and proximal tubular segment, of dog fish (*Mustelus canis*), with proximal, distal segment, and glomerulus, and toadfish (*Opsanus tau*), with proximal segment only, all concentrate mercury to a high degree.

2. High mercury U/P ratios in the toadfish indicate mercury excretion by a secretory mechanism. Excised flounder tubules will not concentrate phenol red if the animal has previously been injected with a toxic amount of chlormerodrin. Accumulation of mercury is also noted under certain conditions in the bile, especially in the dogfish, but is not seen in either stomach or gills.

3. Evidence is reviewed that in many animal species mercury accumulation is greatest in the proximal tubular segment.

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FURTHER EVIDENCE OF THE DESTRUCTION OF BIVALVE LARVAE BY BACTERIA[1]

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Loosanoff (1954) has discussed the significance of large-scale culture of larval oysters and hard clams (*Venus mercenaria*) for experimental purposes and potential commercial use. It has been found that various organisms—the fungus *Sirolpidium*, for example—can destroy larvae in such cultures. However, evidence that bacteria are injurious has been largely circumstantial. Davis and Chanley (1956) showed that larval mortality was decreased at times by the use of various antibiotics. Walne (1956, 1958) found that antibiotics brought about increased spatfall of European oysters and parallel decrease of the bacterial population in his culture vessels.

Davis (1950, 1953), as part of feeding experiments, fed fourteen species of bacteria (including a mixture of *B. coli* and a bacteriophage) to oyster larvae. There was no evidence that any bacteria were of value as food. The four species used in the first experiments (*Vibrio marinofulvus, Micrococcus maripuniceus, Bacillus imomarinus,* and a red sulfur bacterium) were harmful at (unspecified) high concentrations, but not at low ones. However, three phytoflagellates studied produced the same results. In the second experiments, larvae fed bacteria (at unspecified concentrations) died within eleven days, while unfed controls grew slightly. Apparently mortality was not catastrophic. In this connection, observations by ZoBell and Feltham (1938) are significant. They found that adult mussels survived and grew when fed 10^8 to 10^9 washed bacterial/ml. once a day for nine months. Thirty-one clones were used. However, when a non-toxic peptone was added to water containing mussels, the animals died when the concentration of bacteria was only of the order of 10^5/ml. They suggested that metabolites in actively growing cultures were responsible, but it is possible that a different flora was selected by the addition of fresh nutrient solution. Brisou (1955) points out that *Pseudomonas*-like organisms are common in living bivalves. Takeuchi *et al.*, (1957) reported a high mortality of adult *Ostrea gigas* caused by or associated with a bacterium of the genus *Achromobacter*.

This report presents evidence that two clones of bacteria isolated from an infected hard clam (*Venus mercenaria*) larva destroyed healthy larvae, while other clones did not under similar experimental conditions. In the final experiments larvae were reared under aseptic conditions up to the time of exposure to known bacteria, thus excluding the possibility that contaminating microorganisms were the direct cause of death, while the bacteria were secondary invaders.

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MATERIALS AND METHODS

Isolation and growth of bacteria

Bacteria were seen "swarming" about moribund clam larvae in a laboratory culture having heavy mortality. One larva was transferred to a tube of sea water broth (essentially medium STP of Provasoli *et al.,* 1957), and the resulting mixed flora was subcultured daily. Pour and streak plates were made on the second day. Although most colonies appeared to be of two types, twelve obviously different clones were isolated. Ten other clones were isolated from contaminated algal cultures (*Monochrysis lutheri* or *Isochrysis galbana*) or from filters through which laboratory sea water was passed. Cultures of mixed bacteria were obtained by adding raw sea water to sterile broth.

Bacteria were grown at 28.5° C. for about 24 hours, with resulting concentrations of most clones of the order of 10^9/ml. STP broth was used in some of the first studies, but the medium was later standardized to ⅔ strength (Difco) nutrient broth made with sea water. In Experiment 2(2) bacteria were also grown in a clam broth made by autoclaving a minced adult hard clam in its own volume of sea water and decanting the supernatant. Suspensions of bacteria were diluted and samples killed and stained with I_2-KI and counted in a Petroff-Hausser chamber.

Filtrates of bacterial cultures (Experiment 2(3)) were prepared by drawing a few milliliters through sterile ultra-fine fritted glass filters. Filtrate was proved sterile by plating and inoculation into broth.

Bacteria were killed (Experiment 2(4)) by heating to 85° C. for five minutes. Cultures so heated did not grow upon subculture or streaking .

The concentration of bacteria to be used in the final experiments was estimated from measurement of concentrations in preliminary studies, which were of the order of 10^6/ml., and from observations made at various times during 1957–1958 of concentrations in apparently healthy larval clam and oyster cultures. In plate counts from 24–48-hour larval cultures (made on STP agar, ZoBell's No. 2216 agar (ZoBell, 1941), or ⅔ strength nutrient agar) bacterial concentrations were 10^5–10^6 per ml. However, counts of some of these cultures with a Petroff-Hausser chamber and dark field or phase contrast illumination yielded numbers of motile or clearly recognizable bacteria about an order of magnitude higher. (Concentrations in freshly changed cultures were of the order of 10^3–10^4 per ml., determined by plating.) Walne (1958) measured 10^4–10^5/ml. in 24-hour laboratory cultures of European oysters by plating on ZoBell's No. 2216 agar counted at 48 hours. It may be assumed that the actual concentrations were an order of magnitude higher.

Bacterial concentrations used in the final experiments were 10^6 to 10^7 per ml., provided from dense liquid cultures. Dilution made carryover of nutrients insignificant. (Because larvae had been observed to survive exposure to bacterial concentrations of the order of 10^8 per ml., this also was tried in Experiment 1. It was anticipated that larvae might be resistant to the bacteria because of pretreatment with antibiotics.)

Preliminary assays with non-aseptically-reared larvae

Bacterial cultures were assayed by adding 1- to 4-ml. aliquots yielding 10^6 to 10^7 bacteria per ml. to liter cultures of healthy larvae in freshly changed filtered sea water. (Methods of handling larvae are cited in a later section.) Larvae were maintained at a concentration of *ca.* 10/ml. at 24° C. and fed bacteria-free *Isochrysis galbana, ca.* 5×10^4/ml.

After 18–30 hours larvae were concentrated by screening and swirling and examined in a Sedgwick-Rafter cell with a compound microscope (× 150). Because the basic purpose of these assays was to screen bacterial cultures for obviously virulent strains, quantitative counts of mortality were not generally made. Rough quantitative counts were easily made by counting fewer than ten fields.

It was shown that the sterile nutrient broths were non-injurious to larvae at the concentrations used. This was done by adding sterile broth plus the antibiotic mixtures described below.

In each experiment there were two sets of control larvae; one received only food, the other received also an aliquot of sterile broth. In a few experiments bacteria developing in this latter beaker during the experimental period destroyed the larvae. These experiments were discarded even though the same flora might not have developed in the assay beakers.

Assay with aseptically-reared larvae

Straight-hinge clam larvae were obtained free of bacteria by allowing fertilized eggs to develop in solutions of antibiotics shown to be harmless to the animals. In the first experiment 50 mg./l. each of penicillin G (1625 units/mg.) and streptomycin sulfate were used. These concentrations were doubled in the second experiment and 50 mg./l. of chloramphenicol added. Oppenheimer (1955) showed that similar mixtures reduced viable bacteria in sea water to the order of a few per ml. in 24 hours. In our experiments, because adult clams were spawned in sterile sea water and larvae isolated with a micropipette after exposure to antibiotics, chances of contamination were negligible. No bacteria were detected by isolating larvae into sea water nutrient broth. Antibiotics carried over in the isolation technique were insufficient to prevent growth of bacteria.

Procedure

Sea water was autoclaved. Spawning dishes and screens were sterilized with ethanol and rinsed. Adult clams of known sex were washed in warm tap water, rinsed, and spawned by methods described or referred to by Davis (1953). Water was changed if clams did not spawn within a few hours. Fertilized eggs were passed through a 100-mesh screen to free them of feces and pseudofeces, then washed on a 325-mesh screen to free them of excess sperm and to concentrate them. After resuspension and counting, about 2000 eggs were added to 100 ml. of sterile filtered antibiotic mixture in 250-ml. Ehrlenmeyer flasks and kept at 24° C. Twenty-four or 36 hours later the fluid was poured into sterile Petri dishes and apparently healthy larvae caught with a pipette under a dissecting microscope.

Thirty to forty larvae were put into each of a series of 20 × 125-mm. screw-capped culture tubes containing 5 ml. of sterile sea water, and fed bacteria-free *Isochrysis galbana* at a concentration of 10^5/ml. (Control larvae in unchanged water in these tubes went to metamorphosis, but more slowly than those kept in containers as described by Davis and Guillard, 1958.) Aliquots of counted bacterial suspensions were added by pipette.

Motility of larvae could be observed through the test tube walls. At the end of the experiments, clams were again poured into a Petri dish and re-isolated onto a slide, where each was examined with a compound microscope using dark field and phase contrast illumination as necessary.

EXPERIMENTAL

Assay with non-aseptically-reared larvae

Mortality of larvae exposed for 18–30 hours to the mixed culture derived from a moribund clam and to the five succeeding subcultures from it was greater than 90%. "Swarming" bacteria were numerous in moribund animals and were seen swimming freely. No comparable mortality was observed in five trials involving the ten clones derived from contaminated algal cultures or the mixed bacteria resulting from the enrichment of sea water. (Not all clones were included in each of the trials.) Larvae were able to survive in concentrations as high as 10^8/ml. of some of these clones, though they developed abnormally in the higher concentrations. Mortality was negligible in both sets of controls in all these trials.

Two assays were made of the twelve clones isolated from the moribund clam. Ten of these caused no significant mortality, but the other two produced mortality comparable to that of the mixture from which they were derived. These clones, designated 6–1 and 13–1, were the two colony types predominating on agar plates of the mixed culture. Both are gram-negative, non-sporogenous, polar monotrichous rods about 0.75 × 1–2.5 microns in size. Both are halophilic to some extent and have not become adapted to growth on media without NaCl or sea water. The temperature optimum of 6–1 is between 35° and 40° C., while that of 13–1 lies between 25° and 32.5° C. Dr. Einar Liefson of Loyola University has undertaken further study of both strains. He has assigned 6–1 to the genus *Vibrio* and 13–1 to *Pseudomonas*. Some criteria are given in Hugh and Leifson (1953).

Experiment showed that neither 6–1, 13–1, nor the mixture from which they came could injure larvae in the presence of antibiotics. Duplicate larval cultures were inoculated with *ca.* 10^7 bacteria per ml.; to one set was added also 50 mg./l. each of penicillin G and streptomycin sulfate. Animals exposed to bacteria alone were destroyed in 24 hours, while those treated with antibiotics also were indistinguishable from controls exposed to neither or to antibiotics alone. This was done twice with each bacterial culture.

Experiments with aseptically-reared larvae

Experiment 1. This was undertaken primarily to test the method and to determine if bacteria at the concentrations used would kill larvae previously exposed to antibiotics. The experiment proper consisted of eight tubes, as follows:

1. controls, sea water
2. controls, nutrient broth
3. clone 13–1, 10^6/ml.
4. clone 13–1, 10^7/ml.
5. clone 13–1, 10^8/ml.
6. clone 6–1, 10^6/ml.
7. clone 6–1, 10^7/ml.
8. clone 6–1, 10^8/ml.

There were also five tubes in which larvae were grown to metamorphosis.

Examination through the tube walls showed that most larvae were killed in a day in the highest concentration of each clone. The other tubes were examined on the fourth day. Results are summarized in Table I. Larvae exposed to 10^7/ml. of either clone were 95%–100% destroyed by the fourth day. However, 10^6/ml. of 13–1 caused only 10% mortality, while a like number of 6–1 killed 76%. No control animals died.

TABLE I

Mortality of clam larvae caused by three concentrations of clones 6–1 and 13–1 (larvae initially 115–120 microns in size)

Clone	Bacteria per ml.	Time	No of larvae moribund	No. of larvae recovered	% of larvae moribund	Notes
13–1	10^8	1 day	26	27 of 30	96	vela disintegrating
13–1	10^6	4 days	3	29 of 30	10	none over 135 μ
13–1	10^7	4 days	38	40 of 40	95	none over 135 μ
6–1	10^8	1 day	27	32 of 35	84	none over 125 μ
6–1	10^6	4 days	22	29 of 30	76	largest were 160 μ; dead larvae were 120–145 μ
6–1	10^7	4 days	11	11 of ?	100	vela swollen; none over 135 μ
Control	none	4 days	0	30	0	135–160 μ*

* Another group of larvae from the same spawning was kept in polyethylene containers, fed every day and changed every other day. The largest of these larvae were 190 μ.

Bacteria were not counted during or after the experiment. Judging subjectively, there were not as many in the medium at the end as might have expected on the basis of the number added. Some of the moribund larvae were surrounded by "swarming" bacteria.

Experiment 2. The objectives were:

1. To compare the effects of bacteria on larvae kept at three different temperatures; 20, 25, and 30° C. The observation that clones 6–1 and 13–1 grew better at high temperatures than did most other clones isolated suggested this portion of the experiment.

2. To compare the effects on larvae of equal numbers of bacteria grown on two different media—the usual nutrient broth and clam broth. The possibility that bacteria maintained on clam broth might remain more virulent than those maintained on nutrient broth prompted this portion of the experiments.

3. To observe larvae exposed to sterile filtrate of bacterial cultures.

4. To observe larvae exposed to dead bacteria plus their culture broth. Experiments 3 and 4 were to confirm that only living bacteria kill larvae, as suggested by the antibiotic experiment of Section 1. The experiment was carried out in 20 tubes, as follows:

Tube	Temperature ° C.
1. Control, food only	25
2. 6–1, 10^7/ml. (broth-grown)	25
3. 13–1, 10^7/ml. (broth-grown)	25
4. Control, food only	20
5. 6–1, 10^7/ml. (broth-grown)	20
6. 13–1, 10^7/ml. (broth-grown)	20
7. Control, food only	30
8. 6–1, 10^7/ml. (broth-grown)	30
9. 13–1, 10^7/ml. (broth-grown)	30
10. 6–1, grown in clam broth, 10^6/ml.	25
11. 6–1, grown in clam broth, 10^7/ml.	25
12. 13–1, grown in clam broth, 10^6/ml.	25
13. 13–1, grown in clam broth, 10^7/ml.	25
14. 6–1 filtrate, 1 ml. (equivalent to 1 6 × 10^9 bacteria/ml.)	25
15. 13–1 filtrate, 1 ml. (equivalent to 3.2 × 10^9 bacteria/ml.)	25
16. Heat-killed 6–1 (8 × 10^8/ml.)	25
17. Heat-killed 13–1 (8 × 10^8/ml.)	25
18. Larvae in broth alone as sterility check	25
19. Larvae in broth alone as sterility check	25
20. Larvae in broth alone as sterility check	25

Larvae were examined on the fifth day. In Table II and Table III, respectively, are gathered data pertinent to the temperature portion of the experiment and comparison of the effects of bacteria grown on different media.

Mortality was relatively independent of the temperature at which larvae were kept: 73% to 76% of the animals exposed to clone 6–1 were dead, as were 30% to 48% of those exposed to clone 13–1. Maximum mortality in controls was 7%. It should be noted that growth in the 30° C. controls was poor and that food organisms settled in the tubes at this temperature. (Both 20° and 30° controls were bacteria-free at the end of the experiment.)

From Tables I and II it can be seen that there were no consistent differences in mortality caused by bacteria grown on the two different media. Greatly increased virulence would have been evidenced by early heavy mortality easily visible through the culture tube walls. Small but significant differences would not be detected by an experiment such as this.

The hypothesis that only living bacteria destroy larvae was confirmed by the findings in tubes 14 through 17, in which larvae were exposed to filtrate corresponding to more than 10^9 bacteria per ml. or to 8 × 10^8 dead bacteria in their broth. Of 120 larvae, only two were dead, one in tube No. 15 and one in No. 17. How-

TABLE II

Comparison of mortality of larvae exposed to bacteria and maintained at three different temperatures for five days (concentration of bacteria 10^7/ml. Larvae initially 105–110 microns in size)

Clone	Temp. ° C.	% larvae moribund	Average size		Maximum size	
			Living	Moribund	Living	Moribund
6–1	20	73	125	120	145	125
6–1	25	75	130	115	150	125
6–1	30	76	120	120	130	120
13–1	20	30	150	125	165	150
13–1	25	46	145	125	160	140
13–1	30	48	135	120	185	135
None	20	7	145	—	165	—
None	25	0	140	—	165	—
None	30	3	125	—	140	—

ever, larvae grew scarcely at all. They were initially 105–110 microns in size, and increased only to 110–120 microns. (The larger animals seen in control tubes were 165 microns in size, see Table III.) Moreover, larvae exposed to filtrate or dead bacteria were not feeding and were emaciated. This was probably due to the high concentration of metabolites. In the preliminary assays (Section 1) in which larvae were exposed to living bacteria together with antibiotics, those animals exposed to both bacteria and antibiotics were indistinguishable from controls, but the amount of inoculum in this case corresponded only to 10^6–10^7 bacteria/ml. rather than to 10^9/ml.

In both experiments 1 and 2, moribund larvae injured by clone 6–1 differed in appearance from those injured by 13–1. The former often had vela so distended that they exceeded the bodies of the larvae in length. Some vela appeared to be decomposing and occasionally they detached from the rest of the clam. The bodies of larvae often had striations visible going from the hinge towards the velum.

Clams injured by 13–1 usually had vela that were frayed or ragged, with cilia largely or entirely missing. The bodies were emaciated and granular in appearance. Some seemed to be disintegrating from the hinge side towards the velum. Mori-

TABLE III

Comparison of the mortality at 5 days of larvae exposed to bacteria grown in (a) 2/3 strength nutrient broth and (b) clam broth (larvae initially 105–110 microns in size)

Clone	Medium	Bacteria per ml.	% larvae moribund	Average size		Maximum size	
				Alive	Moribund	Alive	Moribund
6–1	nutrient	10^7	75	130	115	150	125
6–1	clam	10^6	40	135	125	150	135
6–1	clam	10^7	97	120	120	120	120
13–1	nutrient	10^7	46	145	125	160	140
13–1	clam	10^6	59	135	120	140	135
13–1	clam	10^7	48	135	120	150	135
Controls		none	none	140	—	165	—

bund larvae in laboratory cultures often fit one of these descriptions, but it is not known if the appearance is in fact correlated with an infecting bacterium.

Discussion

While the mechanism by which clones 6–1 and 13–1 destroy larvae was not studied, evidence available favors the hypothesis that it is by invasion or at least contact rather than by an exotoxin liberated into the medium. The fact that larvae withstood relatively large amounts of glass-filtered, heat-killed, or antibiotic-treated bacterial culture shows that an exotoxin, to be the sole agent, would have also to be extraordinarily unstable. Further, an exotoxin would be expected to have a relatively uniform influence on animals exposed to it, so that larvae would be more or less uniform in size. In fact, however, larvae exposed to bacteria varied considerably in size (Tables II and III), rather in keeping with the hypothesis that they continued to grow until invaded. Finally, there are observations that mortality in large cultures often followed the pattern of an epizootic, and that bacteria were frequently seen swarming in dying or dead larvae.

It is not implied that bacterial metabolites are without influence on larval growth or development. Indeed, the experiment showed that high concentrations stopped growth entirely. It has also been observed that bacterial contamination of algal food cultures sometimes caused abrupt decrease in larval growth rate without immediate extensive mortality. The addition of cultures of bacteria (other than 6–1 or 13–1) often did the same. This depressant effect may well be due to exotoxins.

Strains 13–1 and 6–1 were far more virulent than other bacteria tested and clearly are a hazard to larvae under laboratory conditions. Possibly they were favored by conditions in the larval cultures and finally dominated the flora, at which point the "disease" became obvious. The observation that both strains grow well at temperatures over 30° C., which is relatively uncommon in marine bacteria found locally, supports this idea. At present it is not possible to tell if these bacteria destroy larvae in nature, where both bacteria and larvae are usually less concentrated than they are in cultures. If the "disease" occurs in nature, one would expect to find it under conditions of high temperature and restricted water exchange.

It should be mentioned that the use of antibiotics to control bacteria in larval cultures is apparently more effective when the water supply is changed regularly and two or more antibiotics are used alternately. Probably this prevents the development of a resistant flora. Animals other than bivalves are also benefited; J. Hanks (personal communication) found that larvae of the gastropods *Polinices duplicata* and *P. heros* grew better when penicillin and streptomycin were used in this way. It must be emphasized, however, that the same antibiotics will not prevent growth of injurious bacteria in algal cultures used as food. If impure algal cultures must be used to raise larvae, the algae should obviously be kept at the lowest temperature allowing reasonable growth.

Summary

1. Twelve strains of bacteria were isolated from a moribund *Venus mercenaria* larva in a laboratory culture. These, ten other clones, and mixed bacteria from

sea water were assayed by adding broth culture yielding 10^6–10^7 cells/ml. to beaker cultures of healthy clam larvae. Only the mixed bacterial culture from the moribund larva and two of the 12 strains isolated from it caused extensive mortality. One of the virulent clones (6–1) is a species of *Vibrio*, the other (13–1) is a *Pseudomonas* species.

2. Larvae exposed to virulent bacteria and simultaneously treated with antibiotics were as healthy as controls, showing that active bacteria were necessary to destroy larvae and that metabolites in the bacterial inoculum were not harmful to larvae.

3. Larvae were grown free of contaminating micro-organisms by allowing washed eggs to develop in antibiotic solutions and then isolating straight-hinge larvae by pipette. Either virulent clone (10^6–10^7/ml.) destroyed 10–100% of such larvae. However, exposing the animals to large amounts of glass-filtered or heated broth in which bacteria had been grown (corresponding to *ca.* 10^9 bacteria/ml.) caused no mortality, but retarded growth.

4. Mortality caused by clones 6–1 and 13–1 in groups of clams kept at 20°, 25°, and 30° C. did not vary significantly. However, both virulent clones grow well at 30° C. and higher; thus high temperatures in laboratory larval cultures favor these strains.

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NEUROSECRETORY CELLS IN GANGLIA OF THE ROACH, BLABERUS CRANIIFER [1]

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Neurosecretory cells have been described in many groups of insects (Scharrer and Scharrer, 1954). Most observations have been made upon brain, subesophageal and frontal ganglia, and corpora cardiaca. There is relatively little information concerning neurosecretion by cells within the thoracic and abdominal ganglia of insects. Day (1940) described neurosecretory cells in the abdominal ganglia of Lepidoptera, and Köpf (1957) found neurosecretory cells in the thoracoabdominal ganglion of *Drosophila*. E. Thomsen (1954) found no evidence that neurosecretory materials were accumulating on either side of ligatures of abdominal connectives in *Calliphora* and concluded that these connectives did not carry neurosecretory products.

During an investigation of the functional roles of the neurosecretory materials accumulated within the corpora cardiaca of the roach, *Blaberus* (Özbas and Hodgson, 1958), the search for appropriate controls, *i.e.*, tissues which would contain only non-secretory neurons, prompted an examination of the thoracic and abdominal ganglia of this species. It immediately became apparent that neurosecretory cells of more than a single type were present in all of these ganglia. Since the existence of neurosecretory cells had not been previously reported within the thoracic or abdominal ganglia of Blattaria, and since this order of insects is commonly utilized in experiments on neurosecretion, it appeared worthwhile to make a detailed study of the neurosecretory cells at these hitherto unstudied loci.

The object of the present report is to describe the types of neurosecretory cells present in the thoracic and abdominal ganglia of *Blaberus*, and in so far as it is possible, to trace the movements of secretions produced by these cells, using evidence from ligation and histological studies. The relationships of the neurosecretory cells within the ganglia to secretory cells located elsewhere within the nervous system are also considered.

MATERIALS AND METHODS

The last three larval instars and adults of *Blaberus craniifer* were studied. No significant differences correlated with sex or developmental stages were found among specimens of this group. Only adults were used in ligation experiments because their larger size afforded technical advantages for the operations.

Two fixatives and two stains were used on each type of preparation, so that by

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comparing the results of the various methods it was possible to rule out artifacts produced by any one fixative or stain. Either Bouin or Helly fixatives were used. The stains were the chrome hematoxylin phloxin stain of Gomori (1941), hereafter designated CHP, or the aldehyde fuchsin stain (Gomori, 1950) as modified by Halmi (1952) and Dawson (1953), hereafter designated AF. All sections were cut 5 microns in thickness.

Ligatures were prepared from Clark's Size A black nylon thread. A single strand was teased from this thread and tied around one of the two connectives which pass between the ganglia. The operations were performed through incisions in the ventral sides of unanesthetized animals, and the ligature sites were varied in different animals so that blocks of connectives between each of the thoracic and abdominal ganglia were represented in the total series of 44 operated animals. The operated roaches were maintained in separate pint jars and fed dogfood, fruit, and water, this being the same diet given the standard laboratory colony of *Blaberus*. The animals were sacrificed 5 to 40 days after the operation, the nerve cords dissected out, and histological preparations made by the methods outlined above.

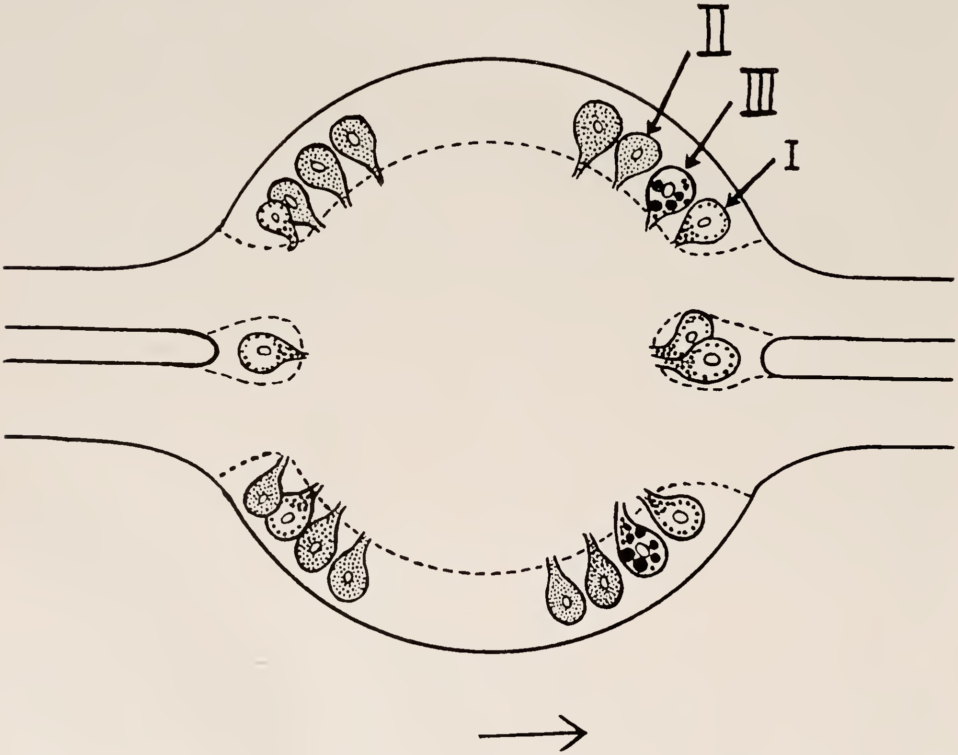

FIGURE 1. Diagrammatic representation of a frontal section through the center of a thoracic ganglion, showing arrangement of neurosecretory cells. The dotted lines separate the peripheral areas which are occupied by cell bodies from the central neuropile mass. Roman numerals designate types of neurosecretory cells—see text. The arrow under the diagram points anteriorly, toward the animal's head.

RESULTS

1. *Neurosecretory cells within the ganglia*

The thoracic and abdominal ganglia were found to contain three types of neurosecretory cells, as shown diagrammatically in Figure 1, and in the photographs A, B, and C of Figure 2. These cells can be differentiated from ordinary neurons by their size, staining characteristics, and the presence of characteristic granules or droplets within their cell bodies and axons. The cell bodies of all three types of neurosecretory cells measure 40 to 60 microns in their longest diameters, 35 to 50 microns in their shortest diameters, and have ellipsoid nuclei measuring 15 to 18 microns in their shortest diameters and 18 to 22 microns in their greatest diameters. The nucleoli are also conspicuous in many of the stained neurosecretory cells. The distinguishing characteristics of the three types of cells are described in the following paragraphs.

Type I (see Fig. 2A). The cell bodies and axon hillocks of these cells contain granules which stain deep purple in AF and dark blue in CHP. There are 5 to 10 such cells in each thoracic and abdominal ganglion, and they are located in the outer portion of the ganglion near the origin of the connectives (see Fig. 1).

Type II (see Fig. 2B). These neurosecretory cells have very small granules distributed uniformly throughout the cell body. The granules stain green with AF and red with CHP. These are the most common neurosecretory cells in thoracic and abdominal ganglia, and there are at least several dozen cells of this type distributed generally throughout the periphery of each ganglion (see Fig. 1).

Type III (see Fig. 2C). Only two neurosecretory cells of this type appear to be located within any one ganglion. They are seen only after Helly fixation and never after use of Bouin solution. The two cells are found in the anterior lateral part of the ganglion, one cell on each side of the ganglion (see Fig. 1). They are characterized by the presence of large droplets which stain orange with AF. These droplets have diameters of 3 to 11 microns. They resemble the "colloid droplets" described by E. Scharrer (1941) within the cells of the preoptic nucleus of the fish, *Fundulus*. There was no indication of cycles of formation or alteration of the droplets in the roach sections such as reported in *Fundulus*.

2. *Observations on normal axons and connectives*

Neurosecretory materials from all three types of neurosecretory cells were observed within axons extending into connectives between ganglia. Small granules, such as found in type I cells, are usually found in no more than one or two axons per section of connective (Fig. 2F). These granules fill the axons, where found, and they appear to move in definite oriented chains.

Secretory material from type II cells is best seen in axons after staining with CHP. This material is the most abundant in axons, and is seen in practically every section of connectives between ganglia. Orange material, presumably from type III cells, was seen in axons of only two animals. The rarity of this material in axons may reasonably be explained by the rarity of the type III cells. When observed in axons, the orange material was not in the form of round droplets, but had the form of elongated rods.

Many glia cells were seen between axons in the connectives and also in the ganglia. These cells were described by B. Scharrer (1939) in the brain, sub-

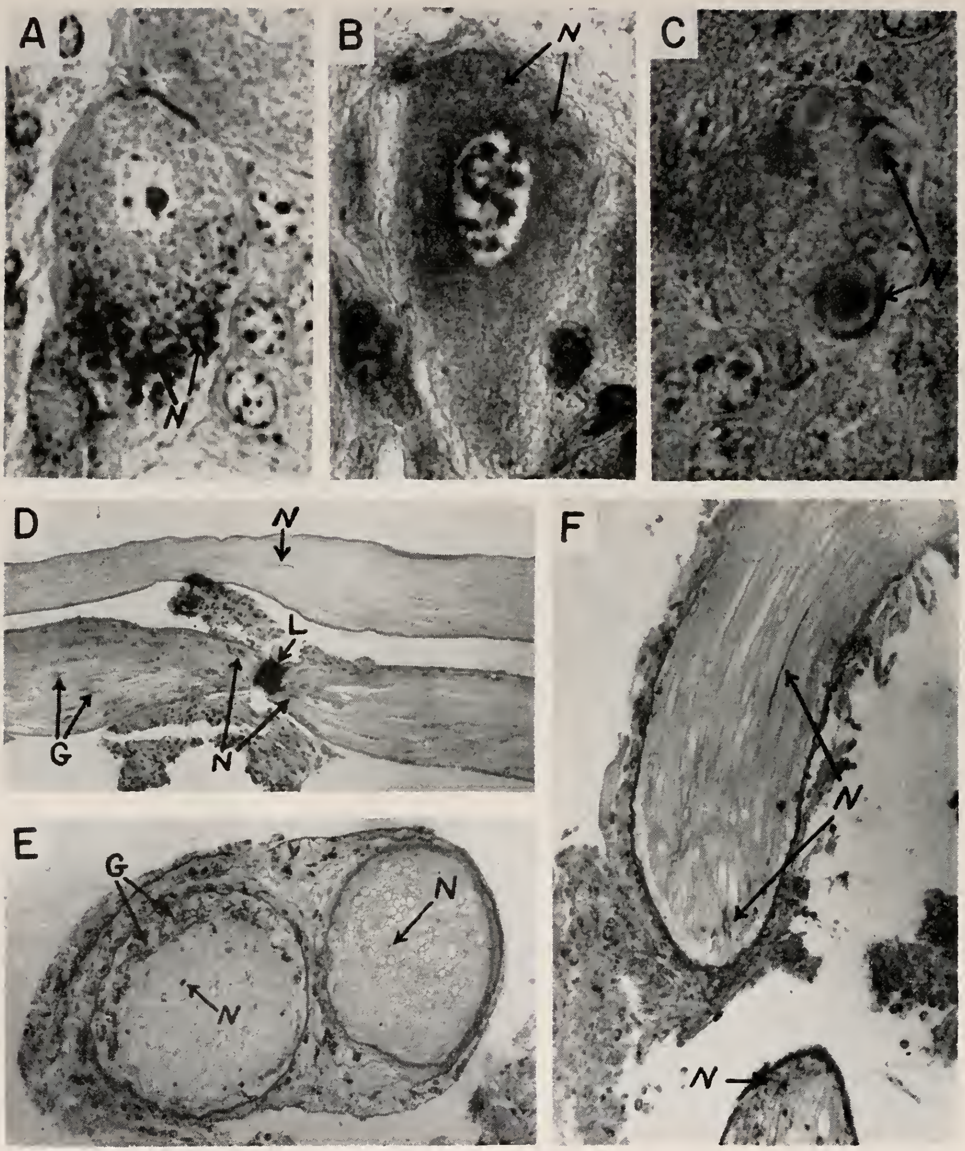

FIGURE 2. Histological preparations. A—type I cell from metathoracic ganglion of adult male (Helly fixation with AF), 1080 ×; B—type II cell from prothoracic ganglion of adult male (Bouin fixation with CHP), 1080 ×; C—type III cell from mesothoracic ganglion of adult female (Helly fixation with AF), 1080 ×; D—frontal section through connective between subesophageal ganglion and prothoracic ganglion (adult male, ligation on animal's right side). Seven days after operation. (Helly fixation with AF), 72 ×; E—cross-section anterior to ligation in left connective between prothoracic and mesothoracic ganglia, control side on right, ♀ adult 20 days after operation (Helly fixation with AF), 144 ×; F—frontal section of left side connective ligated and broken between subesophageal and prothoracic ganglia, ♀ adult, 10 days after operation (Helly with AF). N—neurosecretory materials. G—glia cells. L—ligature.

esophageal ganglion, and connectives of *Periplaneta*. The glia cells are spindle-shaped, have elongated nuclei, and contain gliosomes staining deep purple with AF and deep blue with CHP. In some cases, particularly when the nucleus of the glia cell is not seen, the glia cells might be confused with neurosecretory particles inside connectives, but the latter are in much longer chains and are larger particles than the gliosomes. Roles as supporting cells have been ascribed to the glia cells (B. Scharrer, 1939).

3. *Ligation experiments*

Since ligation of nerves containing neurosecretory axons has been shown to result in accumulations of neurosecretory materials on those sides of the ligations where the neurosecretory materials originate (E. Thomsen, 1954), ligation experiments were used to determine the directions in which the neurosecretory materials were moving. The ligation technique has been described above. In a total of 44 ligation operations, 4 animals died between 10 hours and 12 days after the operation. In 24 cases, the ligated connective was found broken and the nylon thread found in the hemolymph in the vicinity of the operation site. In 16 cases, the connective was not broken and the thread was found still in place (see Fig. 2D).

Whether or not the connective was broken, portions of connective both anterior and posterior to the break or ligation were swollen, as compared to the corresponding unoperated connectives (see Figs. 2D, 2E). The operated connectives, as studied in histological sections, differed from connectives on the control sides in several other ways as well: (a) more glia cells were present on the experimental side, not only in the broken or ligated tips of the connectives, but along their entire lengths (see Fig. 2D); (b) neurosecretory products of types I and II were accumulated in axons on both sides of the breaks, not only localized at the broken or ligated areas, but in elongated masses accumulated within axons on both sides of the operation (Figs. 2D, 2F); (c) no accumulation of type III neurosecretion was seen, doubtless because of its rarity even in the normal connectives.

It is therefore possible to conclude from the ligation experiments that neurosecretory materials from neurosecretory cell types I and II within the thoracic and abdominal ganglia pass in both anterior and posterior directions through the interganglionic connectives. Secretions from type III cells also pass into the connectives but are present in such limited quantities that their movements have not been intercepted in these ligation experiments.

DISCUSSION

With the steadily increasing numbers of neurosecretory cells being described, particularly as new histological procedures are adopted, it is obviously desirable to prevent unnecessary duplication of terminology. Consequently, a more extended comparison of the neurosecretory cells described here with types already described seems appropriate. The most exact resemblance is between the cells which are here designated type II and those designated type B by Nayar (1955) and Köpf (1957). These cells also appear identical to ones staining pink with CHP in the pars intercerebralis of *Blaberus* (Özbas and Hodgson, 1958). Cells of similar staining characteristics have also been seen in the pars intercerebralis of Diptera and Hymenoptera (E. Thomsen, 1954; M. Thomsen, 1954). The uniform distribution of granules within the cell bodies of all these neurosecretory cells and their staining characteristics strongly suggests that they might properly be considered as homologous neurosecretory cells in several groups of insects.

Type I cells are most nearly comparable with, but not identical to, the cells designated type A by Nayar (1955) and Köpf (1957), and previously seen by other workers in the pars intercerebralis of many insects (Scharrer and Scharrer, 1954; De Lerma, 1956; Dupont-Raabe, 1956; Özbas, 1957). The similarities between type I and type A cells consist of the shapes of the cells and their staining properties with CHP. Nayar describes cytoplasm filled with neurosecretory material in type A cells, but in the preparations of thoracic and abdominal ganglia, the neurosecretory material is always near the edge of the cell body. This arrangement of granules within the cell body resembles that described by Scharrer (1955) in the "castration cells" of the subesophageal ganglia of *Leucophaea*. Nayar noted no selective staining of type A cells with AF. Although the AF stain used here is a later modification of the AF staining technique used by Nayar, typical A cells were seen in the pars intercerebralis of *Blaberus*. Cells of identical staining characteristics, but having cyclic secretory activity, were found in the subesophageal ganglion of *Blaberus* during the present study; these cells have been described earlier by B. Scharrer (1941). The distinction between type I and Type A cells must, therefore, be a real one and not a dissimilarity caused by differences of staining technique.

The type III cells described in this report have not been previously described in any insect. Although the droplets which they contain resemble the "colloid droplets" found in certain neurosecretory cells of *Fundulus* (E. Scharrer, 1941), this resemblance cannot be interpreted as implying identity of cell types in such widely divergent groups of animals.

The conclusions from the ligation experiments are contrary to those drawn from ligation experiments on abdominal cords of *Calliphora* by E. Thomsen (1954). However, the *Calliphora* ligations were performed only as controls for other experiments and the results were not studied histologically. Since few axons in the abdominal connectives carry neurosecretory materials, it is not surprising that Thomsen did not find accumulations of neurosecretions in the ligated abdominal connectives which would be seen by inspection of the whole live connectives. The small quantity of neurosecretory material which would be detected in unligated abdominal connectives probably also explains why none was detected when observations were made upon whole central nerve cords using darkfield illumination (M. Thomsen, 1954).

Attempts to detect effects of neurosecretory products from thoracic and abdominal ganglia upon spontaneous electrical activity of *Blaberus* nerve cords *in vitro*, similar to the effects of neurosecretion from the corpora cardiaca (Özbas and Hodgson, 1958), were unsuccessful. This may be due to the small amounts of neurosecretory materials present even in whole thoracic and abdominal ganglia. Moreover, the most abundant type of neurosecretory cells in these ganglia, type II, is relatively scarce in the pars intercerebralis, and all evidence seems to indicate that it is not their secretion which accounts for the effects of corpus cardiacum extracts upon spontaneous nerve activity (Özbas and Hodgson, 1958).

What may be postulated, then, as a normal function of the neurosecretions produced in the ganglia and passing anteriorly and posteriorly through the interganglionic connectives? Although the histological sections were always examined with such a possibility in mind, no clues were found in the form of any areas where neurosecretions from the ganglia were normally being accumulated. It must be assumed that the sites of release of the neurosecretory products are widely scattered throughout the central nervous system, regardless of where their effects are ultimately exerted. Concerning this problem of the normal functions of the neurosecretory cells here described, the present observations can really only be an introduction.

This work was done in the Department of Zoology, Columbia University. The author wishes to express her thanks to Professor E. S. Hodgson for his help and encouragement during the course of the investigation and for his help in preparing the manuscript.

Summary

1. Three types of neurosecretory cells are found within the thoracic abdominal ganglia of the roach *Blaberus craniifer*. These three cell types may be characterized as follows: type I—contains granules staining deep purple with aldehyde fuchsin, the granules being distributed around the periphery of the cell body and in the axon hillock; type II—the most common neurosecretory cell in the ganglia, contains very small granules staining red with chrome hematoxylin and phloxin, the granules being distributed throughout the cell body; type III—the rarest of the neurosecretory cells observed within the ganglia, contains large droplets staining orange with aldehyde fuchsin. The possible identities or homologies of these cell types with others previously described in insects are discussed.

2. Secretory products from all three types of neurosecretory cells have been found within axons in the connectives between ganglia.

3. Ligation of the connectives between ganglia reveals that at least neurosecretory products of cell types I and II move in both anterior and posterior directions from the ganglia in which they are produced. Neurosecretion from cells of type III is very rarely seen in connectives and, consequently, its direction of movement could not be established.

4. No areas of accumulation for any of the neurosecretions were found within the central nerve cords, and the normal functions of these secretions are not known.

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EXPERIMENTALLY INDUCED RELEASE OF NEUROSECRETORY MATERIALS FROM ROACH CORPORA CARDIACA[1]

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Although the pars intercerebralis-corpus cardiacum system of insects has been extensively used for experimental analysis of neurosecretion (B. Scharrer, 1954; Wigglesworth, 1954), there are relatively few data to indicate the conditions under which this system normally releases neurosecretory substances during the life of the animal. Variations in the amount of neurosecretory materials within the corpora cardiaca are known to occur according to the age and the physiological conditions of insects (B. Scharrer, 1952; Wigglesworth, 1954). Variations in the potencies of corpora cardiaca extracts in affecting central nervous activity, as tested by the method of Özbas and Hodgson (1958), have recently suggested that corpora cardiaca from roaches which have been extensively handled or subjected to prolonged surgical procedures contain significantly less neurosecretory material than normal. An analogy with the secretion of adrenalin during the response of mammals to stress situations is further suggested by the isolation from corpora cardiaca of a substance with adrenalin-like effects upon roach and frog hearts (Cameron, 1953; Unger, 1957).

The present experiments were designed to test the hypothesis that neurosecretory materials are released from the corpora cardiaca of the roach when the animal is hyperactive or experiences conditions resembling those which produce symptoms of stress in mammals. Another objective of this work has been to determine whether experimentally induced "stress" conditions produce histologically detectable changes in the neurosecretory cells of the pars intercerebralis or other changes within the brain.

MATERIALS AND METHODS

The roach *Blaberus craniifer* was the experimental animal. Each experimental group consisted of adult females which had undergone their last molt on the same day. This selection was made because some males lack one of the neurosecretory products always observed in corpora cardiaca of adult females of this species (Özbas and Hodgson, 1958), and in order to have the experimental animals as uniform as possible.

Experimental treatments of the roaches consisted of administering electrical shocks to the animals or forcing them to be hyperactive. Ten-volt electrical shocks of 5 milliseconds duration each were administered from an electronic square wave stimulator at the rate of twenty shocks per second. Steel electrodes (size 0 insect pins) were inserted into bilaterally symmetrical positions in the roach's head,

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[1] This investigation was aided by a U. S. Public Health Service Grant (No. E-2271) to Dr. Edward S. Hodgson.

near the medial margins of the compound eyes, or else one electrode was inserted into the right lateral portion of the third abdominal segment and the other electrode inserted into the left lateral portion of the fifth abdominal segment. The shocks thus administered represent approximately the minimum amplitude, duration, and frequency of electrical stimulation to which these roaches gave behavioral responses consisting of abdominal movements and movements of the appendages. Locomotion was prevented during the administration of shocks by pinning each roach through the edges of its pronotum to a dissecting board. Some control animals, hereafter designated C1, were pinned to the board without electrodes, while others, designated C2, were pinned and also had electrodes in the head and abdomen but received no shocks.

Sustained hyperactivity of *Blaberus* was produced by placing the roach within a glass jar and giving the jar a quick shake so that the roach was turned upside down. This posture invariably caused the roach to execute violent leg and wing movements until it had turned itself over, whereupon the jar was immediately shaken again so as to invert the roach and initiate its struggles again. Each animal's activity was sustained by repetition of this treatment throughout the desired length of time.

Control and experimental animals were killed by decapitation at various intervals after treatment, and the entire head (including the corpora cardiaca) of each roach was immediately fixed with either Bouin's or Helly's solution, as specified below. Five-micron sections were cut and stained with either the chrome hematoxylin phloxin stain of Gomori (1941), hereafter designated CHP, or the aldehyde fuchsin stain (Gomori, 1950) as modified by Halmi (1952) and Dawson (1953), designated AF. Since the numbers of animals in each experimental group varied according to the numbers of synchronously molting females available, and their treatments varied according to the information sought, each experimental series will be described separately.

RESULTS

Series I consisted of 6 roaches tested 31 days after molting. Two of these were used as controls (C1 and C2), treated in the manner described above. Both controls were pinned to the dissecting board for 15 minutes and sacrificed immediately thereafter. Animal No. 3 had electrodes in its head only, and received shocks for one minute; animal No. 4 also had electrodes in the head only, but received shocks for 15 minutes; No. 5 had electrodes in the abdomen and received shocks for 15 minutes; No. 6 had one set of electrodes in the head and another set of electrodes in the abdomen, and it received shocks through both sets of electrodes for one hour. All of the animals in this series were fixed in Bouin's solution within one minute after the end of treatment, and the sections were stained with CHP.

The sections revealed marked differences in the amounts of neurosecretory materials within the corpora cardiaca of these animals. The corpora cardiaca of the controls (both C1 and C2) had large amounts of neurosecretory materials staining dark blue and pink. These neurosecretory materials were distributed throughout the central parts as well as the peripheral regions of the corpora cardiaca. Less of both pink and blue staining materials could be seen in sections from animals No. 3, but the most striking results were obtained in the cases of animals 4, 5, and 6. These three roaches had very little of either the pink or blue staining materials remaining in the corpora cardiaca, and most of the traces were found in the peripheral regions of the glands, especially near the aorta. These results indicate a significant loss of both kinds of neurosecretory materials from the corpora cardiaca during the experimental shock treatments lasting 15 minutes or longer.

Since there is some variation in the initial amounts of neurosecretory materials within the corpora cardiaca of different animals, and even in the amounts observed within adjacent 5-micron sections from the same gland, the interpretation of an apparent partial decrease in neurosecretory content of glands from one animal alone, such as No. 3 from this series, would be questionable. The differences between the two controls and the animals receiving shock treatments for 15 minutes or more are very large, however, not only in the total amounts of neurosecretory materials within the glands, but also in the distribution of the materials as described above. To this evidence must be added the results from the other experimental series also.

Series II consisted of 6 animals tested 49 days after molting. The tests were designed to check the reproducibility of the experimentally induced decrease of neurosecretory substances found in Series I, and to determine the rate of restoration of the depleted substances within the corpora cardiaca. Two control animals (C1 and C2) were treated exactly as those in Series I. Each of the other 4 animals received shocks for 15 minutes through electrodes in the abdomen, thus duplicating the treatment given animal No. 5 of Series I. The 4 experimental animals in Series II were sacrificed at the following intervals after the end of their shock treatments: one minute, one hour, 6 hours, and 24 hours.

Since total amounts of neurosecretory materials were of concern in this series, the AF stain, following Helly's fixative, was chosen as the most convenient way of analyzing the results. Allowing for differences in the histological technique, and the fact that Series I animals had undergone their last molt more recently, the control animals in Series II did not differ greatly from the controls in Series I with respect to the amounts of neurosecretory materials within the corpora cardiaca. The controls of Series II had, if anything, slightly more neurosecretory material than the Series I controls which were tested closer to their time of molting. The distribution of the neurosecretory materials within the corpora cardiaca was also similar to that observed in the controls of Series I.

Animals 3, 4 and 5 of Series II had very little neurosecretory material within the corpora cardiaca, the amounts and distribution being approximately the same as found in animals 4, 5 and 6 of Series I. (Photographs A and B of Figure 1 show typical sections through the corpora cardiaca of a control (C1) and animal No. 3 from Series II.) This confirmed the previous conclusion concerning the effects of the shock treatments lasting 15 minutes. The results from this series were inadequate, however, for determining the possible rate of restoration of the neurosecretory substances within the glands. Animal No. 6 had more neurosecretory material in the corpora cardiaca than could be found in the glands of other experimental animals of this series, but the amount was still far less than in the controls. Unfortunately, not enough suitable animals were available to permit more extensive tests concerning this point, but clearly the time required for refill of

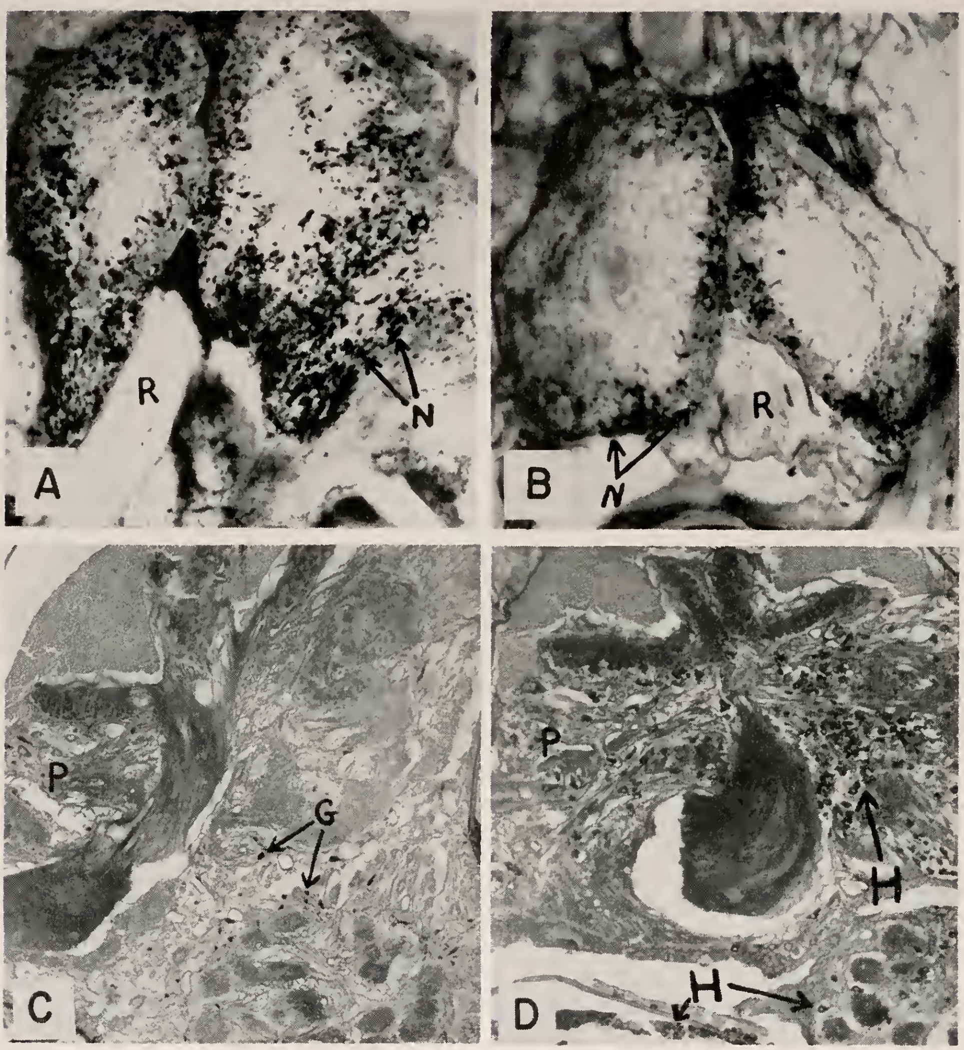

FIGURE 1. Photomicrographs of typical histological sections; all were fixed with Helly's solution and stained with aldehyde fuchsin. A—cross-section of corpora cardiaca from control animal (C1 of Series II), 144 ×; B—cross-section of corpora cardiaca from animal (No. 3 of Series II) which had received abdominal shocks for 15 minutes, 144 ×; C—cross-section of brain of an untreated control (C1 of Series III), 72 ×; D—cross-section of brain of an animal (No. 5 of Series III) which had been hyperactive for one hour, 72 ×. G—glia cells. H—hemocytes. N—neurosecretory materials. P—pars intercerebralis (containing neurosecretory cell bodies). R—recurrent nerve.

the glands to a condition resembling that of the controls must be measured in days, or possibly in weeks, rather than hours.

Series III consisted of 5 animals tested 42 days after molting to determine whether forced activity of the roaches would produce effects upon the corpora cardiaca similar to the effects of electric shocks. Two animals were sacrificed without any experimental treatment to serve as controls; when stained with AF, their corpora cardiaca did not differ significantly from those of the controls in Series II. The experimental animals were sacrificed immediately after various periods of forced activity: one minute, 15 minutes, and one hour. After one minute of activity, no significant reduction of neurosecretory material within the corpora cardiaca was observed. There were, however, marked reductions of neurosecretory materials in the corpora cardiaca of the animals active for 15 minutes and one hour; their corpora cardiaca appeared similar histologically to those from animals given shocks for 15 minutes or longer. These results were interpreted as evidence that "stress" situations other than those caused by electric shock treatments could also induce release of neurosecretory materials from the corpora cardiaca. Certain other effects of the periods of forced activity are presented later in this section.

Series IV consisted of 6 animals tested 18 days after molting. Since Özbas and Hodgson (1958) have shown that corpus cardiacum extracts depress spontaneous nerve activity in roach nerve cords, this series was used to determine whether corpora cardiaca retain their potency for affecting spontaneous nerve activity when the glands are taken from animals which have been hyperactive. Corpora cardiaca were removed from two control animals which were not given any experimental treatment. These extracts were tested in two lots of two glands each, and it was found that they depressed spontaneous nerve activity in roach nerve cords in essentially the same manner as has been previously described by Özbas and Hodgson (1958). Extracts of corpora cardiaca from the other three animals were similarly tested after the animals had been forced to keep active for periods of 15, 60, and 120 minutes each. The glands were removed immediately after the periods of forced activity. None of the extracts of corpora cardiaca from these three experimental animals caused any significant changes in spontaneous nerve activity in the nerve cords, thus showing a clear difference from the controls and confirming the effects of the forced activity upon the neurosecretory content of the corpora cardiaca, using an entirely different method from the histological analysis previously employed.

Since the neurosecretory substances dealt with in this study come to the corpora cardiaca from cell bodies located within the protocerebrum of the roach's brain, sections were also cut through the brains of roaches in Series I through III. These sections were studied particularly with regard to any histological changes in the neurosecretory cells of the pars intercerebralis which might have resulted from the various experimental treatments. The amounts of neurosecretory materials within the cell bodies of these neurosecretory cells varied so much from cell to cell in both control and experimental groups that no significant changes could be attributed to the experimental treatments. (See photographs C and D of Figure 1 for the location of the neurosecretory cells.)

In sections of the brains of some experimental animals, there was one difference from the controls which was very striking—this difference being the presence of blood cells distributed throughout the brains of the experimental animals. This was observed in brain sections from all three experimental animals which had undergone periods of hyperactivity in Series III. At least several hundred blood cells could be seen within the brain of the roach which had been kept active for one hour (see Fig. 1 D), and it was estimated that between one and two hundred blood cells were present in the brains of the two animals kept active for shorter periods. (Exact counts are difficult to make in these cases because the blood cells are sometimes tightly clustered and the same cells may be seen in more than one serial section.) A similar invasion of the brain by blood was also observed in sections of the brain of the one roach in Series II which was sacrificed immediately following 15 minutes of abdominal shocks. Although only 16 blood cells were counted within the brain tissue of this animal, the presence of even a few blood cells within the roach brain must be regarded as significant because no blood cells were ever observed in the brains of any of the control animals similarly stained with AF. No blood cells were seen in material from Series I (stained with CHP), and the absence of blood cells in the other experimental animals of Series II suggests that the invasion of the brain by hemocytes is a temporary one, possibly lasting even less than an hour, although this is really a separate problem which cannot be analyzed adequately from the present results. Some of the other problems raised by this movement of hemocytes will be discussed below.

Discussion

The experimental induction of the release of neurosecretory materials reported here is analogous to the results obtained in several other cases involving both arthropods and mammals. Kleinholz and Little (1949) found that asphyxia, like injection of eyestalk extracts, produced hyperglycemia in the crab *Libinia*. It was later proven conclusively that the mediation of the sinus gland within the eyestalk was essential in such cases of induced hyperglycemia in various crustaceans, and that many experimental treatments, including crowding, handling, and anesthesia of the animals, were also effective upon the sinus gland (presumably causing the gland to release stored neurosecretory substances), thereby producing hyperglycemia (Kleinholz, Havel and Reichart, 1950). The imposition of stress or injury to the animal appears to be a common feature of these experimental treatments affecting the sinus glands (Carlisle and Knowles, 1959).

An analogous case involving the rat has been reported by Rothballer (1953). Release of neurosecretory materials from the neurohypophysis of the rat was brought about by application of painful stimuli to the experimental animals, and there were indications that even handling the rats might result in loss of neurosecretory material from the neurohypophysis. Here, too, the imposition of stress would appear to be a common feature of the different treatments affecting the neurosecretory center. For similar reasons of convenience, the term "stress" is useful to indicate a common feature of the stimuli applied to roaches in the present experiments—that is, these stimuli would be expected to produce discomfort, pain, fatigue, or exhaustion, and to elicit rapid secretion of hormones from the adrenal medulla of a mammal. No identity of the mechanisms of response to stress in mammals and invertebrates is meant to be implied, however.

The exact mechanism linking the electrical stimulation or forced hyperactivity of the roaches and the release of neurosecretory materials from their corpora cardiaca is unknown. In the case of the electrical shocks, particularly in view of the magnitude of the shocks and their application directly within the head in some of the experimental animals, it might be reasonably argued that the shocks were directly stimulating the axons or cell bodies of the neurosecretory cells supplying the corpora cardiaca. Potter and Loewenstein (1955) have demonstrated the conduction of action potentials along neurosecretory cell axons following electrical stimulation in the fish *Lophius*. Knowles, Carlisle and Dupont-Raabe (1955) used electrical stimulation to elicit the release of a chromactivating substance from a crustacean neurosecretory system *in vitro*. The assumption that arrival of nerve impulses, either normally or experimentally initiated, at the ends of the axons of neurosecretory cells would lead to release of neurosecretory substances from such cells is compatible with much contemporary thought concerning the release of neuroendocrine substances (Welsh, 1959).

In the case of hyperactivity involving no electrical stimulation, other mechanisms must be involved. There is already abundant evidence from studies on other neuroendocrine systems that the controlling factors may be quite complex and may exert their actions through more than one intermediary mechanism (Scharrer, 1959), even though transmission of nerve impulses along axons of neurosecretory cells may be the ultimate trigger mechanisms for release of neurosecretory substances from such accumulation centers as the corpus cardiacum. It is unfortunate that the operation of severing the neurosecretory axons between the brain and the corpora cardiaca prior to the periods of forced activity, which might otherwise be expected to test the importance of the innervation of the corpora cardiaca, is an operation which necessitates considerable handling and operative trauma to the animal. In itself, this procedure would probably affect the amounts of neurosecretory materials within the glands, as well as deprive them of their source of supply of neurosecretory materials.

Only very tentative hypotheses can be offered as to the role of these processes in the normal life of the animal.The nature of the experimental treatments which cause the release of neurosecretory materials from the corpora cardiaca suggests that the release of such materials may be part of the normal reactions to stress. It is already known that corpus cardiacum extracts increase the frequency of the insect heart beat (Unger, 1957) ; they also decrease, sometimes after an initial transient increase, the amount of spontaneous activity in nerve cords *in vitro* (Özbas and Hodgson, 1958), and probably other metabolic effects remain to be discovered. Although certain adrenalin-like effects of corpus cardiacum extracts (Cameron, 1953) further suggest an analogy with the secretion of adrenalin during the response of mammals to stress situations, it remains to be determined whether the compounds being released from the corpora cardiaca during experimental conditions such as used in the present study actually bear any chemical relationship with adrenalin. An inclusive interpretation of these diverse effects would be particularly aided at the present time by studies of the effects of stress on central nervous activity, heart function, etc., in the intact animal. The invasion of the brain by blood cells, which was unexpectedly found to follow certain experimental treatments, has not been previously reported. In staining characteristics, the invading hemocytes resemble those within blood sinuses (one of which is shown in the lower left corner of Fig. 1 D). The hemocytes do not, however, exactly resemble any of the types commonly described, but variations from species to species, and with different stains, make such precise identifications difficult even under the best of conditions (Munson, 1953). The hemocytes were not localized in any one part of the brain, and no visible damage to tissues of the brain appeared to follow the experimental treatments; these facts would appear to rule out simple hemorrhage or phagocytic action as an explanation for the distribution of the hemocytes. There is some evidence that hemocytes store, transport, and transform nutritive materials (Munson, 1953), and such might be their functions during the experimental treatments administered in the present cases. Further studies on this problem are planned.

The authors take pleasure in thanking Dr. Berta Scharrer for her helpful discussions of this work and critical reading of the manuscript. The responsibility for any errors and for the conclusions, however, is entirely our own.

SUMMARY

1. Neurosecretory materials within the corpora cardiaca of adult female roaches (*Blaberus craniifer*) were studied histologically, using chrome hematoxylin and phloxin or aldehyde fuchsin stains.

2. Both the neurosecretory substances which are stained dark blue with chrome hematoxylin and those stained pink by phloxine are markedly depleted in the corpora cardiaca following administration of electric shocks for periods of 15 minutes or more to the heads or abdomens of the roaches.

3. Forced hyperactivity of the roaches, when continued for periods of 15 minutes or longer, also causes a marked decrease in the same neurosecretory materials within the corpora cardiaca.

4. Following periods of forced hyperactivity of the animals, there is also a loss of the potency of extracts prepared from their corpora cardiaca, when such extracts are assayed for their ability to depress spontaneous activity in roach central nerve cords *in vitro*.

5. It is suggested that the release of neurosecretory substances from the corpora cardiaca may be a part of the roach's response to stress situations.

6. Hyperactivity of the roaches and, to a lesser extent, electric shock treatments, result in the invasion of all parts of the brain by blood cells. The significance of this phenomenon is not known.

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EXAGGERATED ELEVATION OF THE FERTILIZATION MEMBRANE OF CHAETOPTERUS EGGS, RESULTING FROM COLD-TREATMENT[1]

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There are many phenomena associated with elevation of the vitelline membrane after activation of invertebrate eggs; these have long been of interest to embryologists. Recently, Costello (1958a) has summarized much of the earlier work, and has contributed new observations concerning the behavior of the vitelline membrane and the jelly material which is secreted by the egg of *Nereis limbata* after fertilization. These studies involved the centrifuging and subsequent treatment of *Nereis* eggs with alkaline sodium chloride (pH 10.5), which had earlier been shown by Costello (1945) to be effective in producing exaggerated membrane elevation and denuding of the eggs. The method is based upon one first described by Hatt (1931) and subsequently modified by Novikoff (1939) for the eggs of *Sabellaria vulgaris.*

Costello (1958a) interpreted his results to indicate that centrifuging of the *Nereis* egg resulted in a displacement of the cortical jelly-precursor material, so that upon subsequent activation such centrifuged eggs had an asymmetrical perivitelline space which was widest at the centrifugal pole. The jelly-layer, which forms external to the membrane, was also asymmetrical and thickest at the same area. When centrifuged unfertilized *Nereis* eggs were treated with alkaline NaCl, there was a retention of the jelly beneath the vitelline membrane and formation of an asymmetrical perivitelline space.

Activation of the egg of *Chaetopterus pergamentaceus* is not accompanied by cortical changes of so obvious a nature as those reported for *Nereis* by Lillie (1911), Novikoff (1939) and Costello (1949, 1958a). There are, however, inconspicuous rhythmic contractions of the vitelline membrane of the *Chaetopterus* egg, beginning about 20 minutes after fertilization, which were described briefly by Lillie (1906) and in more detail by Pasteels (1950). It was suggested by Costello (1958a) that (page 180), "These might be occasioned by rhythmic release of small quantities of colloidal material in progressive waves sweeping over the egg surface from an origin at one pole or the other." Evidence which offers support for this hypothesis has been obtained during the course of experiments involving the treatment of *Chaetopterus* eggs with low temperature.

Brief preliminary accounts of some of this work have been reported (Costello and Henley, 1949; Henley and Costello, 1949; Henley, 1958).

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METHODS

Eggs and sperm of the polychaete annelid *Chaetopterus pergamentaceus* were obtained and handled by the methods outlined by Costello *et al.* (1957). The general plan of the experiments involved the following procedure: Two to five minutes after insemination, the experimental eggs were, for those series which involved temperature shock, transferred to six-inch fingerbowls containing pre-chilled (2–3° C.), freshly filtered, aerated sea water; the fingerbowls rested in a nest of cracked ice contained in a large fingerbowl, which in turn was kept in the refrigerator. In the series of experiments which did not involve temperature shock, the eggs (contained in a six-inch fingerbowl of freshly filtered, aerated sea water at room temperature) were gradually chilled by placing the fingerbowl in a larger dish of cracked ice which in turn was placed in the refrigerator. Usually a period of about 60 minutes was required for the temperature of 2–3° C. to be reached in those experiments which did not involve temperature shock.

Appropriate controls were kept for all series; these dishes were kept surrounded by running sea water.

At the end of the treatment period, the culture dishes in all series were removed from the refrigerator and ice-bath to the sea water table, and allowed to return gradually to room temperature, which varied from 20 to 24.5° C. during the various periods of the experiments. Egg-samples were removed and studied at frequent intervals during and following the warming-up period. No temperature shock was involved during the post-treatment period for any of the experiments.

Counts were made to determine the approximate time of 50% cleavage in experimental and control egg-populations, so that a quantitative measure could be obtained of the cleavage delay brought about as a consequence of treatment. All observations and photographs of living eggs and embryos were made without the use of coverslips; drops of culture fluid were examined in uncovered Columbia watchglasses.

For some series, fixed and stained whole-mount preparations were made of control and experimental eggs. These were prepared according to the method described by Henley and Costello (1957), being fixed in Kahle's fluid and stained in Harris' acid haematoxylin, or by the Feulgen technique. Most of the results reported here, however, are based upon observations of living embryos and larvae.

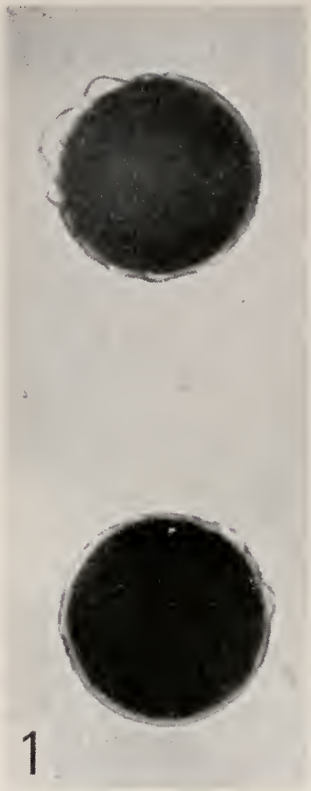
Photographs of living and fixed eggs were made using a Leica camera with Micro-Ibso attachment.

A total of over 30 experiments was performed, during the course of several summers at Woods Hole.

RESULTS

Membrane elevation

The most striking single result of cold-treatment of *Chaetopterus* eggs is the markedly asymmetrical exaggeration of vitelline membrane elevation, which occurs during the period of gradual return of the eggs to room temperature (Figs. 1–10). This process begins rather slowly and, for the first 10 minutes or so (after moderately short periods of treatment), appears to involve no atypical changes in the egg or membrane. A relatively localized shallow crinkling of the membrane then begins at one sector (Fig. 1, lower egg); this is probably, at most, only a slight



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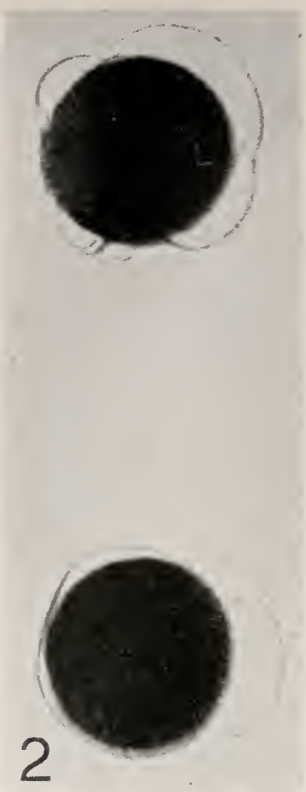

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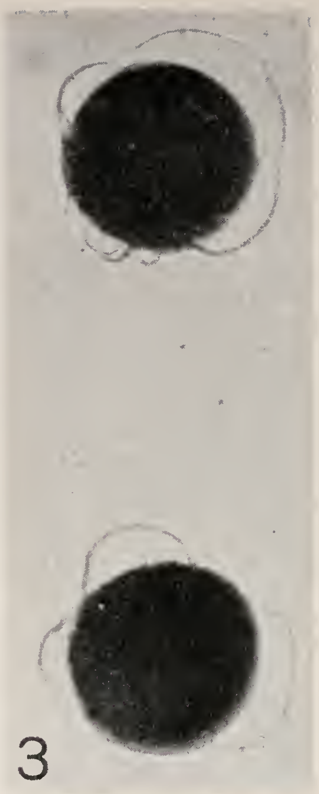

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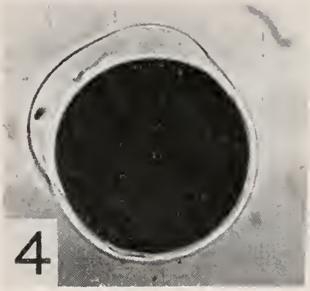

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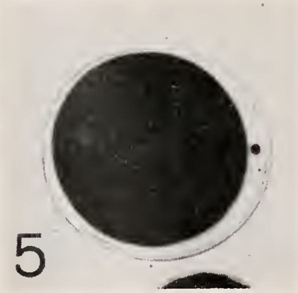

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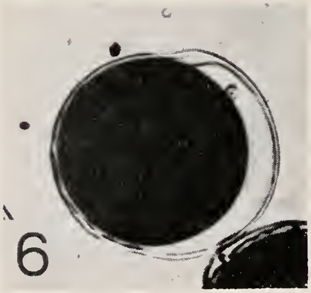

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All photomicrographs are of living, fertilized *Chaetopterus* eggs; the pictures were made with a Leica camera and Micro-Ibso attachment. Magnification of all figures after reproduction: approximately 250 ×.

Figure 1. Eggs cold-treated for 150 minutes with temperature shock and photographed 11 minutes after the end of treatment. In the lower egg, the first area of membrane wrinkling is visible at approximately one o'clock on the egg periphery; the elevation of the entire membrane is still at about the normal height. In the upper egg, the second area of membrane "blistering" has begun to appear, the original area of crenation still being visible at the lower pole of the egg.

Figure 2. Another pair of eggs, cold-treated for 150 minutes with temperature shock and photographed 21 minutes after the end of treatment. Note the enlarged "blistered" areas in the membranes of both eggs.

exaggeration of the normal membrane wrinklings which occur at the vegetal pole, beginning about 20 minutes after insemination (Lillie, 1906; Pasteels, 1950). Within a period of approximately five minutes, a second, much more pronounced, localized area of membrane elevation appears in the treated eggs (Fig. 1, upper egg). This second region is the one from which subsequent exaggerated membrane elevation proceeds. It is, from its first appearance, quite distinctive and is characterized by a more blister-like configuration of the membrane (Fig. 2). It appears to bear no constant spatial relationship to the original wrinkled vegetal pole area from which, as aforesaid, it is entirely separate.

During the course of the next few minutes, additional deep folds may appear in the membrane (Fig. 3); eventually these become contiguous and by 35 minutes after the end of treatment (approximately equivalent to 40 minutes after insemination) the membrane is smoothly and very exaggeratedly elevated from the egg surface (Fig. 10, upper egg). The course of membrane elevation during the period from 28 minutes to 33 minutes after the end of treatment is shown in Figures 7–10. These pictures show clearly that once the process of exaggerated membrane elevation begins, it proceeds rapidly (see also Figures 2 and 3, photographs taken one minute apart).

The ultimate exaggerated elevation attained may be represented by Figure 10 (top), shown 33 minutes after the end of treatment. The elevation of the membrane is quite markedly asymmetrical, the point where it is nearest the egg surface representing the sector which was first wrinkled (not that second center from which the process of elevation proceeds). Even here, however, at the point of closest approach to the egg surface, the elevation of the membrane is much wider than normal. The asymmetrical nature of this elevation is a characteristic and consistent result of cold-treatment.

Comparison of Figures 1 (lower egg) and 10 (upper egg) will reveal the magnitude of the exaggerated membrane elevation; although Figure 1 represents a treated egg, the elevation of the membrane has not yet proceeded beyond the normal stage.

It is important to note that except for the first area of shallow membrane wrinkling, the subsequent stages are probably quite separate and distinct (in degree, at least) from the normal membrane shape changes described by Pasteels (1950). His description of the initial crenated area in an egg 20 minutes after insemination

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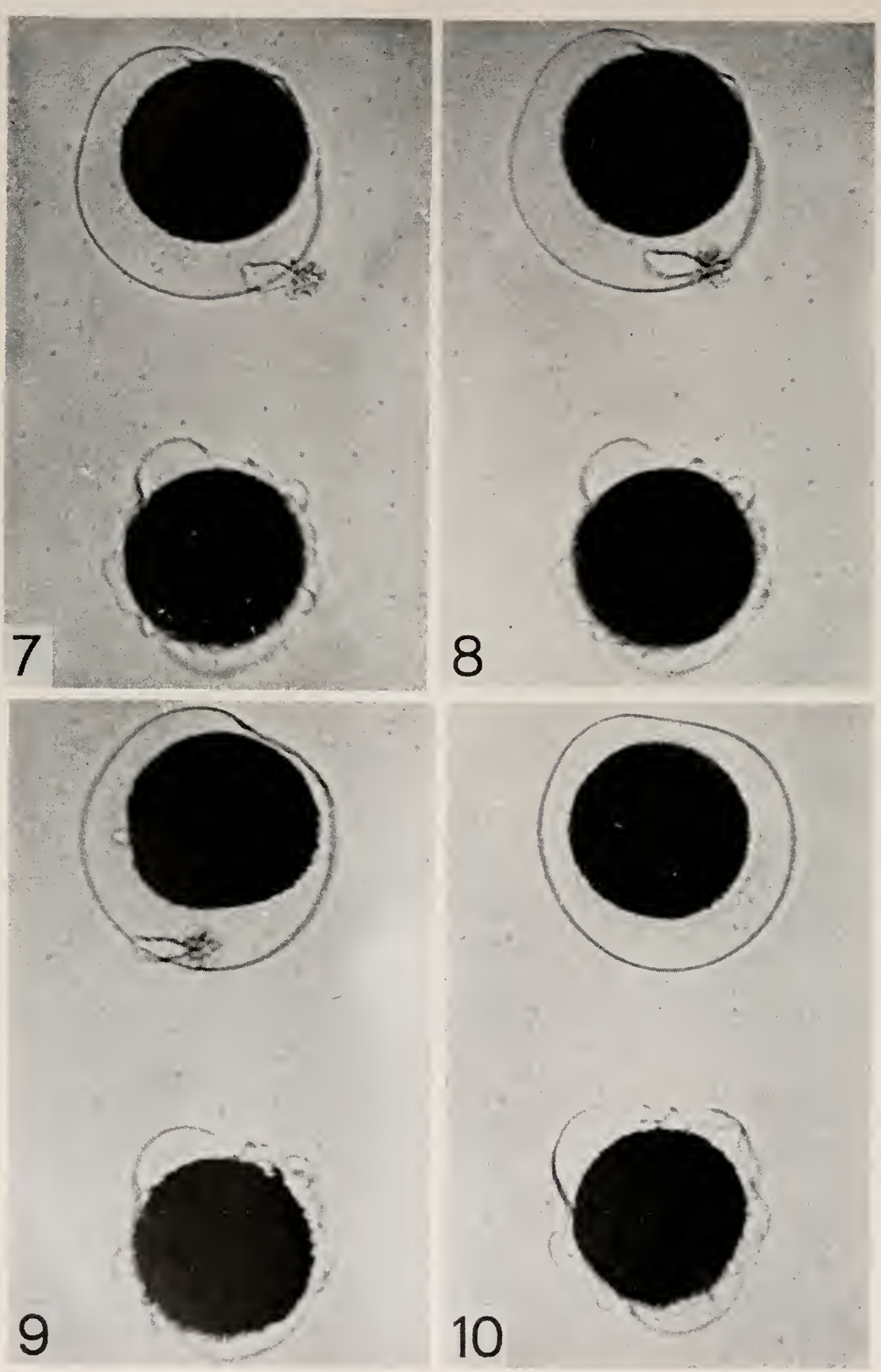
FIGURE 3. The same pair of eggs shown in Figure 2, photographed 22 minutes after the end of treatment (one minute after the photograph of Fig. 2). Note that two additional localized areas of exaggerated membrane elevation have now appeared in the lower egg.

FIGURE 4. Egg cold-treated for 6 hours without temperature shock and photographed immediately after the end of treatment. In this case, the process of exaggerated membrane elevation has begun considerably sooner after treatment than usual. The localized character of the elevation and the double nature of the membrane are apparent.

FIGURE 5. Another egg from the same treatment group as that shown in Figure 4, but photographed 50 minutes after the end of treatment. The double nature of the exaggeratedly elevated membrane is visible. The small globule between the egg surface and the membrane at the right is apparently a polar body.

FIGURE 6. A third egg from the same experimental group as those shown in Figures 4 and 5, photographed 50 minutes after the end of a 6-hour cold-treatment. The membrane has remained closely apposed to the egg surface around approximately one-half of the egg periphery but is exaggeratedly elevated around the other half. The double nature of the membrane is clear.



Figures 7–10.

(at an undesignated temperature) as having a "finement plissé" appearance is very accurate, and his Figure A shows that the subsequent normal wrinklings and shape changes are likewise quite shallow. We have repeatedly confirmed his findings in the study of normal, control eggs, and they are also illustrated in the photographs accompanying the paper by Harvey (1939).

There is no way of knowing with certainty whether the changes observed in our treated eggs are in any way comparable to the normal wrinklings, since the exaggerated membrane elevation is so drastic as to obscure any crenations of the type seen in untreated eggs. They are not associated with the presence of a coverslip, since all our observations of living eggs were made without the use of a coverslip. Careful study of living eggs, of fixed whole-mount preparations, and of photographs of living eggs has convinced us that the exaggeratedly elevated membrane is double in nature (Figs. 4–6).

Cleavage delay

At room temperatures of 20–25° C., the first cleavage of *Chaetopterus* eggs normally occurs from 40 to 50 minutes after insemination (Costello *et al.,* 1957). In the cold-treated eggs studied here, first cleavage for 50% of the experimental eggs was delayed from 134 to 704 minutes, the magnitude of the delay being in direct relation to the duration of treatment (Table I). As noted below in the section on the role of temperature shock, cleavage delay was much greater in those eggs subjected to temperature shock than in those which were cooled gradually (Table II), in series treated for the same length of time. From cytological study, it appears that the cold-treated eggs did not develop very far after the onset of chilling, until the treatment was ended and a gradual warming process (to room temperature) began. During the five-minute period which intervened between the time of insemination and the time when treatment was begun, the sperm penetrated the egg and, in some cases at least, the male and female pronuclei approached one another. Further development did not occur until the conclusion of the treatment. From the data reported in Table II, it appears that a period of from three to ten minutes was required for resumption of development in all cases where eggs were treated with temperature shock. For those eggs which were not abruptly chilled, there was apparently a continuation of some of the early stages of development, during the start of the cooling process, so that the magnitude of the cleavage delay was sometimes slightly less than the duration of treatment.

It is of considerable interest that even in those eggs treated for 720 minutes (12 hours), development could be resumed after cessation of the cold-treatment. As noted in Table I, the trochophore larvae developing from eggs treated for this period were very abnormal, however.

FIGURES 7–10. Successive stages of a single pair of eggs, cold-treated for 150 minutes with temperature shock. FIGURE 7: 28 minutes after the end of treatment. Note the pronounced asymmetry of exaggerated elevation in this and the succeeding photographs. FIGURE 8: 29 minutes after the end of treatment. The small particle of debris has shifted position in the interval between this photograph and that of Figure 7. FIGURE 9: 30 minutes after the end of treatment. FIGURE 10: 33 minutes after the end of treatment. The upper egg (unconfined by a coverslip) has rolled over since the photograph of Figure 9 was taken. The small piece of debris has been obscured. See text for further details.

TABLE I

The effects of cold treatment (5–12 hours) on fertilized Chaetopterus eggs

Duration of treatment	Effects on membrane elevation	Cleavage delay for 50% of exper. eggs as compared with controls	Other effects
300 minutes	Variable; some exaggeration by 15 minutes after end of treatment	301–312 minutes	Ciliary defects in trochophores. First cleavage products equal in size?
360 minutes	Ca. 90% with exaggerated membrane elevation immediately after end of treatment	320–346 minutes	Abnormal trochophores; ciliary defects; surface blebs
390 minutes	Exaggerated elevation by 45 minutes after end of treatment; eventual denuding of many eggs	429 minutes	First cleavage products equal in size?
420 minutes	94% of eggs with exaggerated elevation within 5 minutes after end of treatment	*	Abnormal, amorphous trochophores
540 minutes	90% of eggs with exaggerated elevation within 25 minutes after end of treatment	590 minutes	
720 minutes	44% of eggs with exaggerated elevation within 23 minutes after end of treatment	704 minutes	Abnormal trochophores; some very small. Ciliary defects

All treatments were begun within 2–5 minutes after insemination. The sea water medium was gradually chilled to a temperature of 2–3° C. (no temperature shock).

* The cleavages in this group of eggs were very abnormal and chaotic, and could not be timed.

Other effects of low temperature on development

The embryos and trochophores developing from the cold-treated eggs exhibited a number of characteristic abnormalities. In many of the cases where prolonged periods of treatment were used, there appeared to be a suppression of polar body formation in at least some of the eggs. This is in accord with the findings of other investigators, for other forms (notably amphibians; see the review by Fankhauser,

TABLE II

Treatments of comparable durations, with and without temperature shock

Duration of treatment	Temp. shock?	Effects on membrane elevation	Cleavage delay
180 minutes	No	None, or at most very slight	162 minutes
150 minutes	Yes	Asymmetrical exaggerated membrane elevation by 15–20 minutes after the end of treatment	152–160 minutes
120 minutes	Yes	Asymmetrical exaggerated membrane elevation by 25 minutes after the end of treatment	134 minutes
. . . . . .	. . .	. . . .	. . . . . .
240 minutes	No	Slight exaggeration of membrane elevation in some eggs	220 minutes
185 minutes	Yes	Many eggs with exaggerated membrane elevation by 45 minutes after the end of treatment	189 minutes

1945). Less drastic exposures to low temperature apparently had no effect on polar body formation, so far as could be determined.

In a few experiments, the two blastomeres resulting from the first cleavage of treated eggs were equal or nearly equal in size. (Normally the AB and CD blastomeres resulting from the first cleavage of *Chaetopterus* and other spirally cleaving eggs are noticeably unequal.) Tyler (1930) also observed equal cleavage after cold-treatment of *Chaetopterus* eggs, but his experiments are not entirely comparable to those described in the present experiments, since he used somewhat higher temperatures, applied later in development and for considerably shorter periods of time. Tyler (1930) discusses the production of double embryos by such treatments; no evidence of double embryos, nor of the alteration of cleavage planes as described by him, was observed in the present study.

The normal egg-shape changes ("pear" and polar lobe stages) were often not clearly identifiable in the treated eggs.

The trochophore larvae which developed from cold-treated eggs almost invariably moved very sluggishly, if at all, and there were apparently severe ciliary defects. Surface blebs were often present on the trochophores. In general, the larvae bore a striking resemblance to those obtained after KCl-treatment by Lillie (1902), and after cold-treatment (10–14° C. for 14 hours) by Lillie (1906). However, we do not think that the trochophores in our cultures resulted from differentiation without cleavage, as Lillie (1906) suggested, since cytological study (see below) revealed that at least a degree of both karyokinesis and cytokinesis had occurred in all observed cases.

There was a wide variety of size among trochophores developing from eggs cold-treated for prolonged periods (12 hours). Some larvae were very small, suggesting that they might have developed from egg fragments. Such fragments, or detached cells from later cleavage stages, were observed in culture dishes of this series.

In almost all experiments there was a high mortality rate among the later embryos and trochophore larvae of the experimental groups.

Denuding of eggs

After many of the longer durations of treatment (6 hours or more), the process of exaggerated membrane elevation continued until there was a bursting of the membrane and denuding of the eggs. This denuding was very similar in course and end-results to that observed after alkaline NaCl treatment of *Nereis* and *Sabellaria* eggs. It is, however, in contrast to the process of denuding which follows alkaline NaCl treatment of *Hydroides* eggs (Costello, 1958b); there appears to be an actual dissolution of the *Hydroides* egg membrane in the alkali. (It is suggestive in this connection that Lillie, 1902, described a similar destruction of the *Chaetopterus* egg vitelline membrane in KCl and $CaCl_2$ solutions.) Subsequent development of the denuded *Chaetopterus* eggs was very abnormal under the conditions of these experiments, but no special efforts were made to cultivate such embryos further. Costello (1945) and others have shown that denuded eggs are extremely sensitive to contact with bare glass surfaces, and coating of the culture dishes with a thin layer of agar in sea water is necessary for the successful maintenance of such embryos.

After less drastic treatments, the asymmetrical exaggerated membranes retained their configuration through at least the first cleavage and denuding did not occur.

The role of temperature shock

Table II describes the results of experiments involving comparable durations of treatment time, with and without temperature shock. Even relatively short periods of cold-treatment (120 minutes) involving rapid transfer of the eggs from an ambient medium at room temperature to one pre-chilled to 2–3° C. were effective in producing a marked delay of the first cleavage, as well as the characteristic exaggerated membrane elevation. In contrast, cold-treatments of as long as 240 minutes without this temperature shock resulted in, at most, very slight effects on cleavage time and on membrane elevation. Thus, in one experiment, a treatment of 180 minutes without temperature shock resulted in a delay of 162 minutes for the first cleavage and produced only slight effects on membrane elevation. A similar treatment of 185 minutes with temperature shock resulted in a 189-minute delay of the first cleavage time for 50% of the experimental eggs (as compared with 50% of the controls); by 45 minutes after the end of treatment, there was a marked exaggeration of membrane elevation in many of the treated eggs.

Temperature shock involves the beginning of action of cold considerably sooner after insemination (five minutes) than the absence of temperature shock, where at least 60 minutes may be required to attain the treatment temperatures of 2–3° C. This suggests that the effective action of cold in producing exaggerated membrane elevation occurs within the first hour of treatment; in a gradually cooling medium, there may be opportunity for at least a semblance of the normal release of cortical material to occur, whereas this release is inhibited almost immediately if the eggs are plunged into pre-chilled sea water five minutes after insemination.

Even treatment without temperature shock, however, results in abnormal trochophores and ciliary defects, despite the fact that there may be little or no membrane exaggeration. The implication here is that the effects on membrane elevation may be very different from those affecting subsequent morphogenesis. The low temperature may thus be said to have both a direct and a delayed type of action.

Study of fixed eggs

In one typical group of eggs, cold-treated for 150 minutes with temperature shock, and fixed 15 minutes after the conclusion of the treatment (170 minutes after insemination), the normal quota of 9 chromosomes at the metaphase of the first maturation division was present and countable in most instances. In some eggs, the sperm nucleus was still visible in the interior of the egg, as a separate entity; in others, approach and fusion of the pronuclei had advanced further, and the male and female components were no longer separable on the basis of appearance. Control eggs fixed at the same time were proceeding from the eight- to the sixteen-cell stage in a normal fashion. Experimental eggs of the same series fixed 205 minutes after insemination (50 minutes after the end of treatment) were at the metaphase of the first cleavage, while control eggs were at the metaphase of the fifth cleavage. Several multipolar spindles were observed among the experimental eggs of this group.

The cortical regions of the experimental eggs fixed 50 minutes after the end of treatment presented a striking picture. There was a marked asymmetry of cortical material, apparent as a more lightly staining peripheral band with its greatest width within one relatively localized sector of the egg circumference. These eggs were fixed, whole, on coverslips and there is inherent in the method a certain minor degree of distortion of the egg shape. However, we observed no comparable asymmetry of the cortical material in control eggs of this or any other series, and therefore suggest tentatively that this may represent the area where cortical colloid material has been released in the observed asymmetrical fashion. The eggs of this group were marked by a high incidence of asymmetrical membrane elevation, which was first apparent about 30 minutes after the end of treatment, or 20 minutes before the eggs were fixed. The asymmetry of the cortical area is not visible in the experimental eggs which were fixed 15 minutes after the end of treatment; these ova, in the living condition, had not yet undergone exaggerated membrane elevation.

In addition to the multipolar spindles mentioned above, a number of other types of cytological abnormality were noted, especially in those eggs which were fixed several hours after the end of treatment. Among the abnormalities were chromosome bridges, lagging or lost chromosomes or chromosome fragments, and unequal division of chromatin material to the two poles of the division figure. All these anomalies are typical of the kinds produced in other material as a consequence of low (and high) temperature, among other agents.

There was some evidence of the production of polyploidy in larvae from cold-treated eggs; although a few of the body cells of trochophores obviously had more than the diploid number (18) of chromosomes present, such duplications of individual chromosomes or sets of chromosomes was apparently not the rule in all cells of a given larva. The chromosomes of *Chaetopterus* are small and tend to be crowded on the spindle, so that counting them is difficult, even in early cleavage stages; to do so is almost impossible in the minute cells of later cleavage stages and trochophores. The observed anomalies of chromatin distribution may have arisen as a result of the polar body suppression mentioned above.

Cytological preparations of advanced cleavage stages of the treated eggs indicate that there was often some suppression of cytokinesis (although karyokinesis had proceeded in a variable fashion). In all cases studied, however, at least some degree of cytoplasmic division had occurred, and there was no evidence of differentiation without cleavage. As would be expected, there was a wide variation in cell size, in the treated embryos and larvae.

DISCUSSION

The possible mechanism of action of low temperature

The characteristic asymmetry and exaggeration of membrane elevation in the cold-treated *Chaetopterus* eggs suggest that cold-treatment may interfere with the gradual and rhythmic release of some substance from the egg surface after insemination, so that a sudden localized release of such material occurs at the cessation of treatment. There may also be a change in the permeability of the vitelline membrane, so that the colloidal material is retained within the perivitelline space, bringing about the observed exaggerated membrane elevation. The asymmetry of this exaggerated elevation could be a consequence of the accumulation of colloidal substance within one relatively localized sector of the egg, or of changes in the egg cortex resulting in release of such a substance in a considerably smaller segment of the egg periphery than is normal. The *Chaetopterus* egg secretes no external jelly after activation, and the substance whose abrupt release brings about membrane elevation in the treated eggs is therefore postulated to be of some other nature. Thus, the situation for the *Chaetopterus* egg differs, in detail at least, from that described for the *Nereis* egg by Costello (1958a).

Some additional evidence for this idea is afforded by observations on the action of gum arabic solutions (in sea water) applied to eggs with exaggerated membrane elevation. In such cases, the membranes promptly collapsed back against the surfaces of the eggs.

Pasteels (1950) has also suggested that there may be a rhythmic and localized release of some sort of material from the cortex of the *Chaetopterus* egg, during the post-fertilization period. His experiments involving treatment of eggs of this form with KCl appear to support such an idea if one assumes that the KCl, like cold, blocks the process of release during the course of the treatment.

Pasteels was able to demonstrate a direct correlation in fixed KCl-treated eggs between areas of abnormal membrane wrinkling, and cortical and membrane alterations of structure and staining capacity. This correlation appears to be comparable to that observed in the present study of fixed and stained cold-treated eggs.

Exaggerated membrane elevation as a consequence of other experimental procedures

Lillie (1902) mentioned briefly the occurrence of exaggerated membrane elevation and dissolution in KCl- and $CaCl_2$-treated fertilized and unfertilized *Chaetopterus eggs*. A more thorough study of the same problem was undertaken by Pasteels (1950), who treated unfertilized *Chaetopterus* eggs with KCl by the method of Lillie. Pasteels illustrates a process of asymmetrical membrane elevation which, in some respects, is reminiscent of that reported in the present study. His Figure B shows that by 40 minutes after the end of KCl-treatment (95% sea water, 5% 2.5 *M* KCl, for one hour), there are the beginnings of an asymmetrical membrane elevation which, at this stage, is similar to that observed after cold-treatment. The subsequent course of events after KCl-treatment, however, is quite different; by 102 minutes after the end of this treatment, there is a very asymmetrical, deeply rugose exaggeration of membrane elevation. Apparently this stage is not succeeded by a smooth state of exaggerated membrane elevation.

Redfield and Bright (1921) reported exaggerated elevation of the *Nereis* egg membrane after various types of irradiation. Following beta or gamma radiation, the elevation was symmetrical; after alpha or ultraviolet treatment, it was asymmetrical. Redfield and Bright state that they did not obtain an increase in the volume of the perivitelline space after the irradiation of eggs such as *Cumingia*, *Asterias*, *Arbacia* and *Chaetopterus*, which do not normally produce jelly as a part of the fertilization reaction. However, Moser (1939), Spikes (1944) and Rustad (1959) reported the asymmetrical elevation of the sea urchin egg fertilization membrane after ultraviolet irradiation of one side of the eggs. It is of interest that x-irradiated unfertilized *Chaetopterus* eggs (treated with 20,000 and 40,000 r and then inseminated) showed no exaggeration of membrane elevation (Henley, 1958). Furthermore, whole-mount preparations of such x-irradiated eggs, fixed at the metaphase of the first cleavage, do not reveal the asymmetry of cortical material reported above for cold-treated eggs. Although this observation is by no means conclusive evidence, it does suggest that the appearance of such cold-treated ova is associated with the postulated abnormal retention and delayed release of cortical material.

As noted in the introduction, alkaline sodium chloride treatment (pH 10.5) results in a drastic exaggeration of membrane elevation in the *Nereis* egg (Costello, 1945; Lovelace, 1949); this exaggeration, unlike that reported here, is symmetrical in nature, except for the circumscribed area of sperm entrance. Alkaline NaCl-treatment of *Chaetopterus* eggs is followed by an extremely rapid elevation of the membrane, which results in denuding of the eggs in less than five minutes. The exact process involved will be described in a later communication; suffice it to say here that the membrane elevation appears to be only superficially comparable to that produced as a result of cold-treatment.

Moser (1939) illustrates asymmetrical (although not especially exaggerated) membrane elevation in saponin-treated unfertilized *Arbacia* eggs.

The chronological relationships of events in cold-treated vs. control eggs

It is interesting to compare the chronological relationships of events in the cold-treated eggs and in the normal, untreated eggs. Pasteels (1950) has described the following series of membrane shape changes and wrinklings in fertilized *Chaetopterus* eggs; he did not specify the room temperature but, from the times noted for several "landmark" events, this may be assumed to have been about 17–20° C., somewhat lower than those prevailing during our experiments.

20 minutes after insemination: There is a vegetal-to-animal pole wave of shallow membrane wrinklings.
23 minutes after insemination: The first polar body is given off; from now until after the second polar body appears, the membrane remains smooth.
30 minutes after insemination: The second polar body is given off.
32 minutes after insemination: A second vegetal-to-animal pole wave of membrane wrinkling occurs.
38 minutes after insemination: "Pear-shaped" stage; there is now an animal-to-vegetal pole wave of wrinkling in the membrane, reversing the first two waves.
42 minutes after insemination: Polar lobe stage; wrinkling of the membrane now occurs in a wave from *each* pole, more or less simultaneously, leaving an equatorial band around the egg free of wrinkles.
66 minutes after insemination: First cleavage begins; the wrinklings of the membrane are accentuated.

Our observations showed that for all experiments, both with and without temperature shock, the greatest incidence of exaggerated membrane elevation occurred immediately before the first cleavage (at a stage corresponding to the polar lobe although, as noted above, the normal shape changes of the egg cytoplasm were often not recognizable in the treated eggs). This is a time, of course, when a number of important events are occurring within the egg, in preparation for the first cleavage. It does not seem unreasonable to suppose that the double wrinkling (animal-to-vegetal and vegetal-to-animal) described by Pasteels as occurring shortly before the first cleavage, or some feature of this phenomenon, may be exaggerated to result in the reported events.

SUMMARY

1. Fertilized eggs of the polychaete annelid, *Chaetopterus pergamentaceus,* were cold-treated for various periods of time, ranging from 150 to 720 minutes, beginning immediately after insemination. Two general methods were employed; in one, the eggs were plunged into pre-chilled, filtered aerated sea water (2–3° C.); these experiments are referred to as involving temperature shock. In the other type, the eggs were gradually chilled to approximately the above temperature. At the end of the treatment period all eggs were allowed to return gradually to room temperature.

2. When eggs were cold-treated, with or without temperature shock, there was a pronounced asymmetrical exaggerated elevation of the vitelline membrane, which reached its greatest incidence about 40 minutes after the end of treatment, or shortly before the first cleavage. This exaggerated elevation continued in some cases after prolonged cold-treatment, so that the eggs were eventually denuded.

3. Most of the cold-treatments used were followed by delays in the first cleavage time for 50% of the experimental eggs as compared with 50% of the control population.

4. In all cases where cold-treatment was initiated with temperature shock, the effects on membrane elevation and cleavage time were much more pronounced than when the eggs were chilled gradually.

5. A number of characteristic morphological and cytological abnormalities were noted in embryos developing from the treated eggs; these included severe ciliary defects, fragmentation of the embryos, lagging or lost chromosomes or chromosome fragments, duplication of chromosome sets and/or individual chromosomes, suppression of polar bodies, and multipolar spindles.

6. It is suggested that the findings reported here afford evidence supporting Costello's (1958a) hypothesis that the rhythmic wrinkling and shape changes reported for the normal *Chaetopterus* egg by Pasteels may be due to the gradual release of some colloidal material from the surface of the egg. This release is considered to be blocked in some manner by the low temperature, and when the treatment is terminated, the release proceeds in a drastic, non-rhythmic manner; it then appears to be coupled with a change in the permeability of the vitelline membrane, so that the colloidal material is retained between the egg surface and the membrane. This brings about the exaggerated membrane elevation observed.

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HISTOCHEMICAL STUDIES ON THE NATURE AND FORMATION OF EGG CAPSULES OF THE SHARK CHILOSCYLLIUM GRISEUM[1]

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Pryor (1940) demonstrated that the ootheca of *Blatta* is composed of a tanned protein similar to that of insect cuticle. Since then a number of authors reported the occurrence of tanned proteins in the cysts of nematodes, egg shells of helminths, setae of earthworm and byssus of *Mytilus edulis* (Ellenby, 1946; Stephenson, 1947; Dennell, 1949; Brown, 1952; Smyth, 1954). In this connection it is of interest to recall the observation of Brown (1950a) that the egg capsules of selachians also give evidence of phenolic tanning although it was believed by the earlier workers to be formed of a material similar to keratin. In the light of these observations it was thought that a study of the nature and composition of the egg capsules of shark would be of interest in itself and for comparison with the egg shells of helminths and ootheca of *Blatta* which have been shown to possess a protein constitution resembling in essential respects that of tanned cuticles of insects.

The materials used in the following study comprise nidamental glands and egg capsules collected from gravid females of *Chiloscyllium griseum*. The staining and histochemical reagents used are mentioned in relevant context. For localization of the oxidase of the egg capsule, the "catechol" technique (Smyth, 1954) was applied. The microchemical and chromatographic procedures employed for the study of the protein of the egg capsule are described in the text.

Egg Capsule—Structure

The egg capsules of sharks and rays have been described by a number of workers (Beard, 1890; Widakowich, 1906; Clark, 1922; Hobson, 1930; Nalini, 1940). The shell material is said to be secreted by the cells of the nidamental gland and the egg capsule is formed in the caudal part of the oviduct. The sequence of events in the formation of the egg case is not known for certain. There is some evidence to suggest that a major part of the egg case is formed before the arrival of the fertilized egg. The egg case with the enclosed egg is later ejected into the sea.

The egg capsules of sharks are more or less rectangular in shape with the corners prolonged into anterior and posterior pairs of horns, but considerable variation exists in shape and size in the different species. There is little precise information regarding the nature of the material composing the egg capsule. The earlier workers referred to it as chitinous but the term as used by them carried no chemical significance. On the other hand Hussakof and Welker (1908) who reported on the chemical nature of the egg case of two species of sharks, suggested that it may be similar to keratin. However, the previous workers are agreed that in all selachians the structure and composition of the egg capsule are identical. Widakowich (1906) noted that in *Scyllium* the egg capsule is formed of a large number of "Platten" which adhere to each other loosely at first and later, especially after contact with sea water, much more closely so that the entire capsule hardens. It has also been observed that the capsule when first formed within the oviduct is white and soft and gradually hardens, undergoing a change in color to brown and later to deep reddish brown. In *Chiloscyllium griseum* the egg capsules taken from the oviducts show a range of coloration varying from very light brown to deep reddish brown. The ridge-like thickening bordering the capsule is more deeply colored than the rest of it.

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[1] This work was carried out in the Department of Zoology, University of Madras. I am thankful to Dr. E. R. B. Shanmugasundaram of the Department of Biochemistry for help and advice in regard to the chemical analysis reported in this paper.

Frozen sections of the capsule wall, which is lightly colored, show an outermost narrow yellowish layer containing dark granular inclusions. Internal to it is a broad region more or less uncolored and characterized by horizontal laminations. On the inner border of the laminated region is a very narrow strip which is yellow colored and apparently homogeneous, similar to the outermost layer (Fig. 1). In the laminated region a central part may be distinguished by its darker shade. The color disappears after treatment with ethylene chlorhydrin and the entire section is marked by a diminution of the dark color. This reaction may suggest the presence of melanin-like substances in the wall of the capsule (Lea, 1945). Further support to the above suggestion is obtained by the results of tests performed on the sections of the capsule wall with hydrogen peroxide and potassium permanganate, both of which produce a bleaching effect (Pearse, 1954).

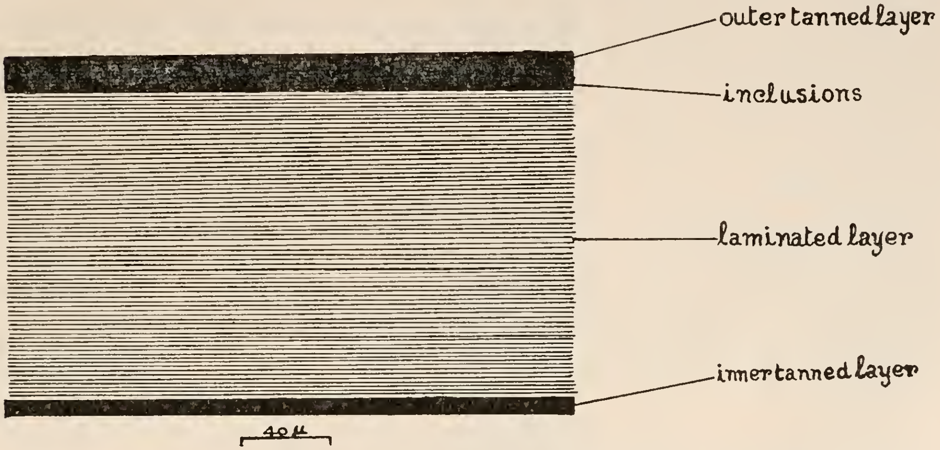

FIGURE 1. Section through the wall of a light colored egg capsule stained with Mallory.

In paraffin sections stained with Mallory, the outer- and innermost layers, which are yellowish in unstained preparations, are colored red with acid fuchsin while the laminated region is stained blue. Similar results were obtained with Masson's trichrome stain; the regions coloring red with Mallory are stained with xylidene ponceau and the laminated region is colored green. With Heidenhain's haematoxylin the outer and inner layers are dark blue while the rest of the thickness of the wall comprising the laminated zone is very lightly or not at all stained. In the regions of the capsule which are reddish brown, the staining reactions are different from those reported above. The central wide laminated region, instead of staining uniformly green or blue with Masson's and Mallory stains, shows red patches filling up the greater extent of this region. Such a change in staining reaction may suggest that the substance originally present has undergone a transformation so as to resemble that present in the outermost layer.

Table I

Responses of egg capsule wall of Chiloscyllium griseum to chemical tests

Test	Egg capsule wall	Outer layer	Middle laminated zone	Inner layer
Millon's	light brown	+	−	+
	deep reddish brown	++	++	++
Xanthoproteic	light brown	+	−	+
	deep reddish brown	++	++	++
Biuret	light brown	−	+	−
	deep reddish brown	−	−	−
Argentaffin	light brown	++	+	++
	deep reddish brown	++	++	++
Ferric chloride	light brown	+	++	+
	deep reddish brown	+	+	−
Ammonium molybdate	light brown	+	+	+
	deep reddish brown	+	+	+
Sudan Black B	light brown	−	−	−
	deep reddish brown	−	−	−
Chitosan	light brown	−	−	−
	deep reddish brown	−	−	−
Pepsin	light brown	−	+	−
	deep reddish brown	−	−	−
Lead acetate	light brown	−	−	−
	deep reddish brown	−	−	−
Sodium nitroprusside	light brown	−	−	−
	deep reddish brown	−	−	−
Dilute mineral acids	light brown	−	+	−
	deep reddish brown	−	−	−
Boiling H_2O	light brown	−	+	−
	deep reddish brown	−	−	−

+ = positive.
− = no apparent effect.

CHEMICAL COMPOSITION

To test the validity of the above assumption, the nature of the principal chemical components of the capsule walls yielding the staining reaction reported above was investigated. It is known from previous work that the major constituent of the shell substance is protein. In the following study a qualitative estimate of the protein constituents was made by color tests and the results are summarized in Table I. It is seen that the entire thickness of the capsule wall is positive to tests for protein. But the light colored capsules differ in some respects from those which are deep reddish brown. It has been pointed out that the capsule when first formed is white and later turns to light brown which gradually deepens to dark reddish brown, the changes in coloration representing different phases in the growth of the capsule. In the earlier phases of growth when the capsule is very light brown, the outer and innermost layers give positive Millon's and xanthoproteic reactions, while the central laminated region is negative to these tests, but positive to biuret test. In the more fully formed capsule, which is deep reddish brown, the central laminated zone also is positive to Millon's and xanthoproteic tests. These changes in the reaction to protein color tests coincide with those in staining reactions with Mallory. Presumably the fuchsinophil substance staining red with Mallory is the same as that giving a Millon-positive reaction.

A positive reaction to Millon's test has been interpreted as indicative of a protein containing the hydroxyl-phenyl group in the molecule, and since tyrosine is the only amino acid containing it, it may be inferred that in the egg capsule Millon-positive sites may contain a protein rich in tyrosine (Pearse, 1954). The coincidence of the Millon-positive regions with those giving positive xanthoproteic reactions supports the above suggestion since the latter test is said to indicate protein containing tyrosine, tryptophane and phenylalanine (Pearse, 1954). On the basis of the positive results obtained with the above two tests, Blower (1951) suggested that the presumptive exocuticle of the myriopods studied by him contains a protein, rich in phenolic groups and which are involved in the tanning of the exocuticle. Similar results have been reported by Krishnan (1956) in the cuticle of *Scolopendra*. In the light of the above observations, a reasonable interpretation of the results of tests on the egg capsule is that when first formed it is constituted of a simple protein positive to biuret test and soon after, the outer and inner layers are modified by the presence of a protein rich in phenolic groups which appear to spread throughout the thickness of the wall. These changes are correlated with a deepening of the color of the capsule and also an increased chemical resistance.

Evidence for Quinone-Tanning

The features noted above recall strongly the characteristic change undergone by the insect cuticle during hardening by tanning (Dennell and Malek, 1955) and appear significant as indicative of a similar tanning process in the egg capsules. Brown (1950b) pointed out that if a structural protein dissolves only in sodium hypochlorite solution and is itself secreted by tissues containing polyphenols, there is circumstantial evidence for quinone tanning. It will be shown in the sequel that both the tests are positive with the egg case material. When small pieces of dark brown capsule wall were treated for varying periods with a dilute aqueous solution of sodium hypochlorite, the color was readily lost and on continued treatment the egg capsule wall was dissolved. Further evidence of tanning is indicated by the presence of a phenol oxidase which is known to be an essential participant of the tanning process in insect and crustacean cuticles (Bagvat and Richter, 1938; Dennell, 1947a, 1947b; Krishnan, 1951). In recent studies on the formation of helminth egg shell, which involves quinone tanning, the oxidase concerned has been demonstrated by the red color produced on incubation with a dilute solution of catechol (Smyth, 1954). The mechanism of the above reaction is due to the oxidation of catechol to quinone and its condensation with the protein so as to produce a tanning effect. This technique was applied to the egg capsules of shark with positive results. Light colored capsule walls when subjected to the catechol treatment changed to a dark red color resulting from the tanning of the protein. Such a color change is less intense with dark colored capsules. That the change in color after catechol treatment is really due to the tanning of the protein may be inferred from the observation that the color is lost on addition of a dilute solution of sodium hypochlorite. Further, sections of material deeply colored by catechol treatment when stained with Mallory show correlated change in staining reaction, the central laminated region being fuchsinophil, so as to simulate the condition of a more full grown and normally reddish brown capsule. It would appear that by treatment with catechol the protein of the central layer is artificially tanned. The color change noted above was inhibited by cyanide in a concentration of 0.001 *M*, suggesting the enzymatic nature of the process and the role of an oxidase in bringing about the tanning effect.

The above observations suggest that the egg capsule after its formation undergoes a process of hardening by phenolic tanning before being ejected into the sea, and this would account for the change of its coloration from light brown to deep reddish brown. The principal participants in the process appear to be a protein probably rich in tyrosine and a phenol oxidase. The results of histochemical tests on the egg capsule material (Table I) indicate an absence of lipids which are usually associated with tanning in the cuticles of insects and other arthropods. Further, although diphenols are indicated in the capsule walls, as may be inferred from positive ferric chloride tests, in the absence of a correlation between their accumulation and the tanning of the protein, their mere presence may not indicate that they are involved in tanning. Their persistence in the outermost layer, where tanning is more intense than in the rest of the thickness of the wall, may militate against the view that they are the tanning phenols. It is suggested that they may be related to the formation of melanin occurring in the capsule walls, for diphenols, indicated by ferric chloride, accumulate in the laminated zone early in the growth of the capsule and their partial disappearance is followed by the occurrence of melanin. This feature, together with the presence of a phenol oxidase in the capsule wall, may suggest the oxidation of phenols to melanin. Since the latter appears even before the onset of tanning in this region it is suggested that the free diphenols may not be directly involved in the tanning process.

Histochemistry of the Nidamental Gland

With a view to investigate further the nature of the tanning process, a study of the mode of formation of the egg shell material was made. It is known from previous work that the materials forming the egg case are secreted by the nidamental gland. The gland is a dilatation of the oviduct at the junction of the caudal and cranial parts comprising a glandular body formed of tubules in a more or less parallel series. The histology of the gland in *Chiloscyllium* shows close agreement with that of *Scyllium canicula* and *Scyllium catalus* (Nalini, 1940). In the anterior part of the gland, distinguished as the albumin gland, the secretory tubules are formed of both glandular and ciliated cells. The secretions are in the form of transparent cytoplasmic granules which appear to be extruded by rupture of the cell walls into the lumen of the gland tube. The succeeding section of the gland, which is distinguished as the shell-secreting zone, is formed of cells whose cytoplasm is packed with granules during the period when egg capsules are being formed. The shell substance appears to be derived from these secretions and in the light of the foregoing observations on the egg capsules, one would expect to find in these cells the constituents of the tanning system. Accordingly histochemical tests for phenols and proteins were applied. The argentaffin, ammonium molybdate and sodium iodate tests for phenols were positive in the cytoplasm of the cells of the shell-secreting zone. Identical regions of the cells were also positive to biuret tests for protein. Malachite green, which is known to show a specificity for proteins involved in tanning of egg shells of helminths (Johri and Smyth, 1956), gave positive reaction in the cytoplasm, the granules taking a vivid green color. The green coloration is said to be due to the dye becoming bonded to the protein. Since the cytoplasmic granules in the cells of this region react positively to both the tests for phenols and proteins, it is suggestive that the substance reacting may be a phenolic protein, similar to that reported to occur in the vitelline gland cells of helminths (Smyth, 1954). Frozen sections of this region of the nidamental gland when treated with a dilute solution of catechol develop readily a brown coloration in the cytoplasm of the cells. This reaction may suggest evidence of the occurrence of a protein undergoing tanning and an oxidase in close proximity to it, responsible for the oxidation of phenols involved in tanning. It appears probable that the oxidase and the substrate are both located in the cytoplasm of these cells.

Nature of Egg Capsule Protein

The foregoing observations indicate that the principal constituent of the egg capsule is a protein secreted by the cells of the nidamental gland, along with an oxidase capable of oxidizing catechol to quinone. In the egg capsules two apparently distinct protein constituents seem to occur, one forming the basal matrix which persists for some time in the central laminated region and the other being a tanned protein which is distinguished from the former by the chemical and staining reactions. In these respects they present very strong resemblance to the basal protein and that impregnating the regions destined to be tanned in the cuticle of insects like *Periplaneta* (Dennell and Malek, 1955). Here the basal protein of the procuticle stains blue with Mallory, is negative to Millon and xanthoproteic tests and lacks chemical resistance, while that impregnating the presumptive exocuticle stains red with Mallory, is positive to Millon and xanthoproteic tests and possesses considerable chemical stability. That the above characteristics of the protein of the presumptive exocuticle may be due to some sort of aromatic bonding is suggested by the observation of Kennaugh (see Dennell, 1958) that the staining properties can be reversed by treatment with Diaphanol which is known to break up the aromatic bonds by oxidation. The change in staining reaction with Mallory from red to blue, reported by the above author, may indicate a restoration of the protein component to its original state, as is found in the untanned endocuticle. In the egg capsule of the shark it is suggestive that the tanned protein is derived from the basal protein such as is found in the laminated region in the earlier stages of capsule formation. If it is so, it may be possible to restore the tanned protein to the original state by breaking up the aromatic bonds as has been done in the insect cuticle referred to above. This was carried out by adopting the method used by Dennell (1958) who following Trim (1941), separated the tanning phenols of the puparia of *Calliphora* using alkaline stannite solution for breaking up the quinone bonds. Accordingly, small pieces of egg capsule material were left in a mixture of 2% sodium hydroxide and stannous chloride at 37° C. for nearly a week, by which time the protein was solubilized. The protein fraction was separated and tested. Unlike the tanned protein it was negative to Millon's test and was easily digested by pepsin-hydrochloric acid and showed a marked swelling in boiling water.

These observations, in addition to suggesting that the tanned protein of the egg capsule may be a derivative of the basal protein, also give some indication of the nature of the protein. The reaction to pepsin and swelling in boiling water are suggestive, especially in the light of the observation of Astbury (1945) that the entire egg capsule of shark yields an x-ray diffraction pattern similar to that of a collagenous protein.

With a view to test further the suggestion made above, a microchemical analysis of the capsule protein was made using a modification of the method of Spencer, Morgulis and Wilder (1937), who applied the above method for a determination of collagen content of the muscles of rabbit. The capsule walls were cut into small bits and cleaned by scraping with a blunt scalpel to remove all adhering tissue. A sample weighing 0.1 gm. was homogenized with an equal quantity of distilled water in a Potter-Elvehjm homogenizer, and the material was placed in a water bath at 100° C. for about 15 minutes along with 10 times its weight of water. This was later stored in a refrigerator, and next day, it was autoclaved for 3 hours at 20 pounds pressure so as to convert collagen, if any, into gelatin. The material was then centrifuged at 4,000 rev./min. for one hour and the supernatant fluid drawn off. An aliquot of this fluid was treated with 3% tannic acid when a copious precipitate was obtained. The above evidence in support of the view that a collagenous type of protein occurs in the egg capsule was checked by a chromatographic analysis of the precipitate. The material was treated with ten times its weight of 6 N HCl in a sealed tube and hydrolysed at 105° C. for 24 hours. The hydrolysate was dried in a vacuum desiccator containing potassium hydroxide and this was used for analysis by partition chromatography, following the capillary ascent method of Williams and Kirby (1948). The hydrolysate was dissolved in a small quantity of distilled water and a 20-μl sample was used for spotting on the filter paper and the chromatogram run with butanol-acetic acid-water as the solvent. Simultaneously a number of chromatograms were run under identical conditions using pure amino acids for purposes of comparison. A solution of 0.1% ninhydrin in butanol was used for spraying. Qualitative analysis of the chromatogram thus obtained shows in general an agreement in amino acid make-up with that of mammalian connective tissue (Bowes and Kenten, 1949), suggesting that the protein in question may be allied to collagen. Further, the pattern of spots was more or less identical with that of a sample of pure gelatin hydrolysed and otherwise treated in the same way as the test material. Most of the amino acids found in the chromatogram of the egg case material correspond to those found in the gelatin.

However, it is seen that the egg case material differs in the absence of hydroxylysine, leucine and valine as well as in the presence of tryptophane. The absence of hydroxylysine may suggest a relationship to elastin, but the occurrence of tryptophane is unusual for a collagenous type of protein. It is possible that its presence may be due to a contaminant. However, it must be mentioned that the amino acid composition of collagen derived from different sources may vary markedly. The collagen of fish skin is known to differ from mammalian collagen in having a low hydroxyproline content while serine, threonine and methionine are in greater amounts (Gustavson, 1956). Such quantitative variations occur not only in those amino acids considered to be characteristic of collagen but also in some of the non-typical residues like tyrosine. A quantitative amino acid analysis is therefore necessary for making a valid comparison. However, the present study is essentially from a biological viewpoint and such evidence as has been obtained is enough to indicate the nature of the material composing the egg capsule. The presence of non-polar amino acids like glycine and alanine, the prominence of proline and hydroxyproline and the comparative rarity of aromatic residues are features of the egg capsule protein, which together are suggestive that it may be allied to the collagen group (Gustavson, 1956).

Discussion

The foregoing observations suggest that the egg capsules of *Chiloscyllium* undergo a tanning process resulting in acquisition of mechanical rigidity and chemical resistance. The process is comparable to that occurring during the formation of ootheca of *Blatta* (Pryor, 1940) but certain differences are significant. Unlike in the insect, here the substrate involved in tanning is a protein without a lipid component. No free diphenol appears to participate in the process. The resultant tanned product is also different from the tough amber colored sclerotin, being only yellowish, and retains a reactivity to stains. The protein constitution of the egg capsule appears to be such that it cannot yield sclerotin after tanning, for it has been observed in arthropod cuticles that unless the protein precursor of tanning is impregnated with a lipid constituent, sclerotin may not be the resultant product. Sclerotin itself has been considered as a lipoprotein subsequently tanned. It is clear that the tanned protein of the egg case is not sclerotin but recalls in its chemical and staining reactions the tyrosine-rich protein precursor of sclerotin, found in the presumptive exocuticle of an inset like *Periplaneta* (Dennell and Malek, 1955) or the so-called pro-sclerotin described by Blower (1951) in the myriopods studied by him. In the above instances the protein in question shows considerable chemical stability even before forming a complex with the lipid participant of tanning and stains red with Mallory, unlike the protein confined to those regions which do not undergo tanning. The chemical stability of the protein has been attributed to the occurrence even at this stage of some kind of aromatic tanning which is distinct from the subsequent tanning of the lipoprotein complex by free diphenols resulting in sclerotin (Dennell, 1958). Such a tanning has been distinguished by the above author as "primary tanning" in contrast to the "secondary tanning" which results in sclerotin. In the absence of the participation of free diphenols "primary tanning" would be presumably by oxidation of tyrosine side-chains of the protein. The tanned protein of the shark egg capsule recalls strongly the product of "primary tanning" in its chemical nature, staining characteristics, possession of resistant qualities and retention of a "tannable" condition having still free amino groups. It seems probable from the observations reported in the present study that the mode of tanning of the egg capsule protein may involve a process of auto-quinone tanning similar to that suggested to occur in the egg shells of helminths (Smyth, 1954).

SUMMARY

1. The egg capsules of *Chiloscyllium griseum*, when first formed in the oviducts, are soft and white and gradually turn light brown to deep reddish brown before being ejected into the sea.

2. Light brown capsule walls show in section an outer and an inner narrow layer apparently homogeneous and yellowish in color while a wide central region is laminated and uncolored. This layer stains blue with Mallory, indicates the presence of a simple protein positive to biuret test and lacks chemical resistance. The outer and inner layers stain red with Mallory and contain a protein which is positive to Millon and xanthoproteic tests indicative of phenolic groups. In deeply colored walls the central laminated layer shows staining and histochemical reactions similar to those of the outer layer.

3. Evidence has been presented indicating that the above changes may be due to the tanning of a basal protein involving a phenol oxidase resident in the capsule wall.

4. The constituents of the tanning system are derived from the secretions of the cells of the nidamental gland. The tanning of the egg capsule protein does not appear to involve free diphenols so that some form of auto-quinone tanning seems to occur.

5. The tanned protein of the egg capsule is unlike the sclerotin of the insect cuticle, but recalls in its staining and histochemical reactions the protein precursor of tanning impregnating the presumptive exocuticle of insects like *Periplaneta*.

6. The nature of the egg capsule protein has been investigated using microchemical and chromatographic methods. From the results obtained it is suggested that it is allied to the collagen group of proteins.

7. The results are discussed.

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THE SIZE AND SHAPE OF METAMORPHOSING LARVAE OF VENUS (MERCENARIA) MERCENARIA GROWN AT DIFFERENT TEMPERATURES

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Among the characters to be employed for identification of lamellibranch larvae, their size at metamorphosis has been suggested by some workers (Lebour, 1938). Other students have maintained, however, that since there may be correlations between size and environmental factors, the size at metamorphosis cannot be used as a criterion for recognition of larvae. Sullivan (1948) thought that this was confirmed for mature larvae of *Mya arenaria* in Canadian waters, which she observed to metamorphose in Malpeque Bay upon reaching a length of 250 microns, as compared with a length of 415 microns reported by Stafford (1912) for larvae of the same clam in the St. Andrews region.

According to Sullivan the difference in size at setting was probably the result of the difference in the summer water temperature at these two places because larvae developing at the lower temperature (St. Andrews) needed to reach a larger size before metamorphosing. This opinion is, indirectly, in agreement with that of Erdmann (1934), who thought that the size of larvae of the European oyster, *Ostrea edulis,* at the time of swarming was regulated by the temperature at which the larvae developed.

So far, no one has offered a definite explanation as to why lamellibranch larvae should reach a larger size if grown in colder water. It seems, however, that this opinion was formed because it is widely believed that, "In many instances, and perhaps as a general rule, the size that an animal attains is greater when it is reared at low temperature" (Coker, 1947, p. 102). Furthermore, since it is known that large individuals of the same species have a relatively smaller surface area than do small ones, the larger size may be considered as an adaptation to an increase in the viscosity of the water which accompanies a decrease in temperature. Examples to support this assumption can be found in numerous papers, including those of Murray and Hjort (1912), and many students of Radiolaria, copepods and certain other forms (Hedgpeth, 1957). Kofoid (1930), for instance, reported that marine protozoa living in cold water grow to much larger sizes than do their relatives existing at higher temperatures.

Aquatic biology also offers many instances in which organisms of the same species grown at different temperatures may show a somewhat different shape. The phenomenon of cyclomorphosis, reviewed by Brooks (1946), is an example. In lamellibranch larvae, of course, no radical changes in structure, as observed in Daphnia populations, can be anticipated. Yet, it has been reported (Jørgensen, 1946, p. 296) that at least in some lamellibranchs, such as *Venus gallina,* "the larval outline varies from almost square to circular." According to the same author the veligers of the common mussel, *Mytilus edulis,* of Danish waters also show a remarkable variability in their shape. The water temperature during development can again be suspected as a factor affecting the shape of the larva.

During the past few years larvae of approximately 20 species of lamellibranchs have been successfully cultured from fertilized egg through metamorphosis by members of our laboratory (Loosanoff, 1954). The data collected during these studies will soon permit us to offer reliable material for recognizing larvae of the species with which we have been working. It will include photomicrographs of the larvae and length-width measurements of their shells from early straight hinge stage until metamorphosis. However, before offering these criteria, it was deemed necessary to ascertain the following possibilities, which could reflect on the reliability of our material:

1) We wanted to know whether, as is suggested by some students, individuals of larval populations grown at relatively low temperatures reach a larger size before metamorphosing than do larvae grown in warmer water. If this is true, special corrections, perhaps as formulae, should be offered to show the relationship between average size at setting and water temperature.

2) Since one of the criteria for recognizing a larva nearing metamorphosis is its dimensions, *i.e.,* length and width, it was necessary to determine whether the length-width ratio is relatively constant or if it changes in conformance with the temperature of the water in which larvae develop.

The questions posed above could be answered only on the basis of well-controlled experiments in which the water temperature was the only factor varied. Obviously, because of the time and efforts required, it would have been difficult to conduct such experiments with larvae of all 20 species of lamellibranchs with which we were working. We decided, therefore, to limit ourselves to observations on one or two species only. This paper is devoted chiefly to a description of the observations on size and length-width ratio at the beginning of metamorphosis of larvae of the hard shell clam, *Venus* (*Mercenaria*) *mercenaria,* developing at different temperatures.

Certain aspects of the studies which provided data for this paper have already been described (Loosanoff, Miller and Smith, 1951). In brief, they consisted of growing larvae of *Venus* (*Mercenaria*) *mercenaria* at constant temperatures ranging from 15.0° to 33.0° C. at intervals of 3.0° C. Since fertilized eggs that were placed in water of 15.0° or 33.0° C. showed abnormal development and heavy mortality, few ever reaching veliger stage, growth of larvae at these temperatures will not be discussed here.

The work was done in winter, the time we find most convenient to control the water temperature (Loosanoff, 1949). Altogether, four experiments were conducted. However, in one experiment one of a pair of cultures grown at 24.0° C. was accidentally lost, while in the fourth experiment, which was conducted during a comparatively warm spell when low temperature was difficult to maintain, no cultures were carried at 18.° C. As is our practice, the water in the culture jars was changed every second day (Loosanoff and Davis, 1950). The larvae were fed a mixture of micro-organisms consisting principally of *Chlorella* sp.

Samples for larval measurements were taken 48 hours after fertilization and every second day thereafter, until metamorphosis. These samples consisted of 50 larvae measured at random from each culture vessel, *i.e.,* 100 larvae from each temperature group. The length represented the greatest distance between the anterior and posterior shell margins, while the width was the distance measured from the tip of the umbo to the middle of the ventral shell margin.

Because the larvae could not be marked individually, their rate of growth and size at setting could not be recorded directly on this basis. For this reason we used two substitute criteria. One was the average length of the larvae on the day the setting was first observed in each culture, and the second, the maximum size of the larvae observed during the life of the culture.

The number of days required for setting to begin at the different temperatures in the four experiments is given in Figure 1. Clearly enough, there were differences between the cultures within the same temperature group and also between the groups carried at different temperatures. The most uniform results were obtained with the 30.0° C. group, where the beginning of setting in the different cultures varied between seven and nine days after fertilization, a difference of only two days. However, the difference between the shortest and longest periods needed for larvae to begin setting became greater in colder water. For example, in the 18.0° C. group the earliest beginning of setting was recorded 16 days after fertilization and the latest, after 24 days, a difference of eight days (Fig. 1).

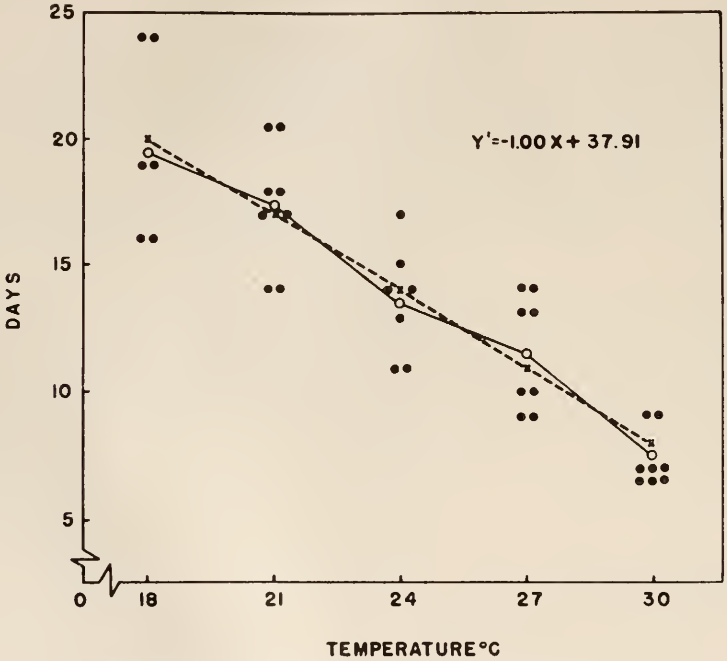

FIGURE 1. Number of days necessary for clam larvae to begin setting in cultures grown at different temperatures. ● = mean of individual culture; o = mean of all cultures grown at the same temperature; x = mean for given temperature predicted from regression line.

An analysis of variance was carried out to test the significance of the differences among the different temperature groups on the number of days required for setting to begin. Since the result was highly significant (beyond the .001 level), separate "t" tests were run for all possible pairs of temperature groups. All the "t" tests were highly significant (beyond the .01 level), with exception of the comparison between the 27.0° and 24.0° C. groups, and between the 21.0° and 18.0° C. groups. These results show, therefore, that a very strong relationship exists between temperature and date of setting, *i.e.*, larvae reared at high temperatures set significantly earlier than those raised at lower temperatures. This conclusion was expressed in the preliminary paper (Loosanoff, Miller and Smith, 1951).

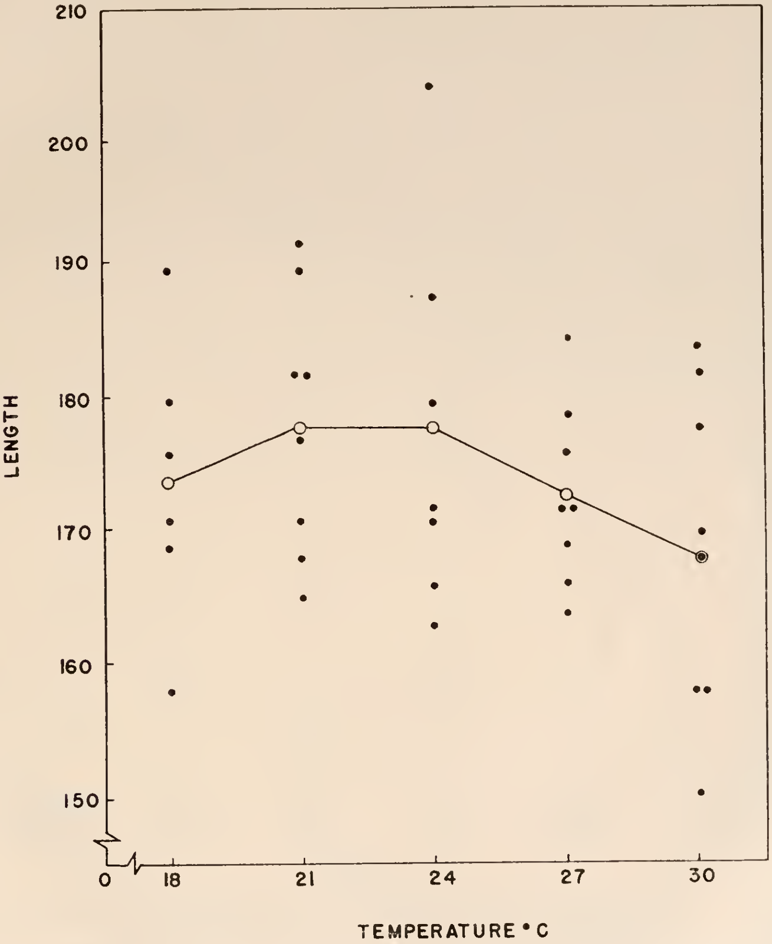

FIGURE 2. Mean length of clam larvae grown at different temperatures on day of beginning of setting. Measurements in microns. ● = mean of individual culture; o = mean of all cultures grown at the same temperature.

Plotting of the dates of beginning of setting in the different cultures against the temperatures showed that the mean number of days for setting to begin for the various temperatures lies on an almost straight line (Fig. 1). Since the line con-

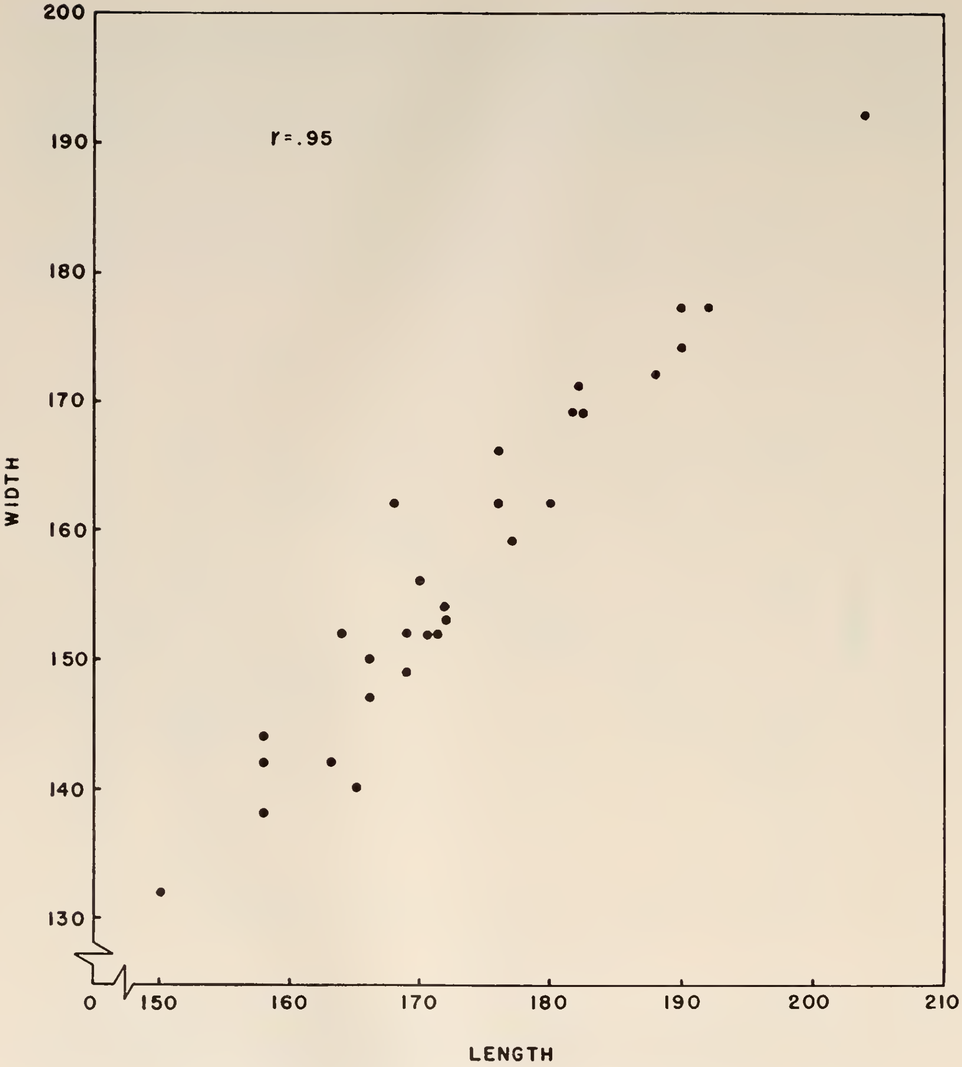

Figure 3. Mean length and width of clam larvae of individual cultures on day of beginning of setting. Measurements in microns.

necting the means of the different temperatures is obviously rectilinear, a regression equation was computed and found to be $Y' = -1.00\,X + 37.91$, where Y' is the predicted setting date and X is the temperature.

Because the regression line is an excellent fit to the experimental data, it is probable that interpolation within the 18.0° to 30.0° C. limits of the experiment can be made with a fair degree of confidence. However, extrapolation beyond these limits is not justifiable. This was shown by our other experiments, which demonstrated that clam eggs placed in water having a temperature of 15.0° or 33.0° C.

did not develop normally. Therefore, since the lineal regression does not hold even for a slightly higher or lower temperature, it cannot be expected to hold for lower or higher temperatures.

An analysis of variance test showed no significant differences among the five temperature groups, with respect to mean length of larvae at date of setting. Thus, although larvae grown at different temperatures required different periods to reach metamorphosis, in all cases they reach approximately the same mean length before setting. This observation indicates, therefore, that there was virtually no relationship between temperature and mean length at date of setting (Fig. 2). Nevertheless, the same figure shows that there was considerable variation in mean length at the beginning of setting among the various cultures within each temperature group.

In our studies we were also concerned with the shape, at metamorphosis, of larvae grown at different temperatures because, as has already been mentioned, the literature contains several remarks concerning variability of shape of larvae of the same species near setting time. Since the simplest method of describing the shape of a larva in mathematical terms for statistical analysis is to indicate its length-width ratio, measurements were made on larvae of all cultures, except those constituting the fourth experiment where no width measurements were taken, and the correlation between the mean length and the mean width of the larvae of each culture, on the day of the beginning of setting, was determined (Fig. 3). The

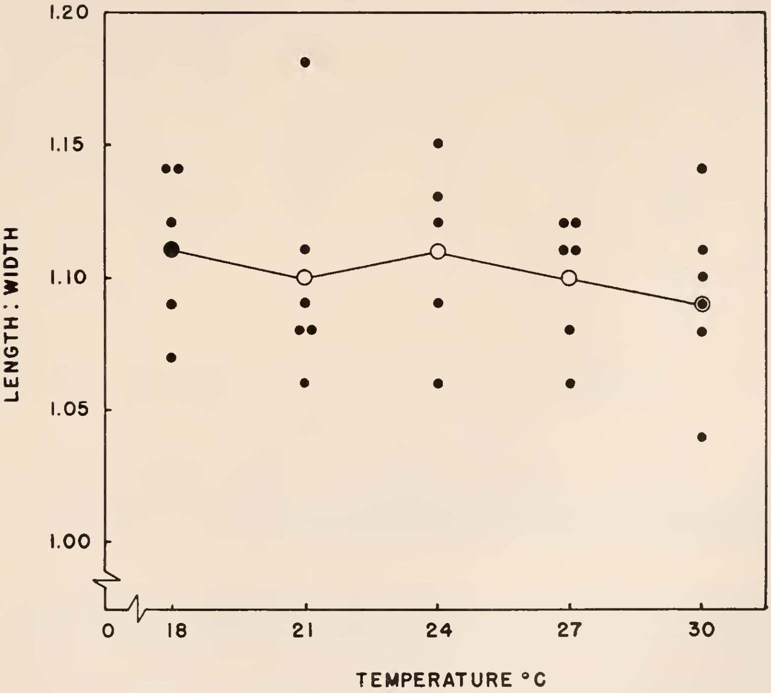

FIGURE 4. Ratio of mean length to mean width of clam larvae of different cultures on days of beginning of setting, in relation to temperature. ● = mean of individual culture; o = mean of all cultures grown at the same temperature.

results indicated this correlation to be so high ($r = .95$) that it seems unlikely that any analysis made using width as a variable would add anything new to that already made with length.

Continuing the analysis of data that might help in discovering the differences in shape of larvae grown at different temperatures, a study was made of the ratio of mean length to mean width at the date of setting. It failed to show the existence of any significant change in the ratio at different temperatures (Fig. 4). Thus, this matter has been satisfactorily solved to assure investigators working with lamellibranch larvae that individuals of the same species display virtually the same shape at metamorphosis, even though they are grown at different temperatures.

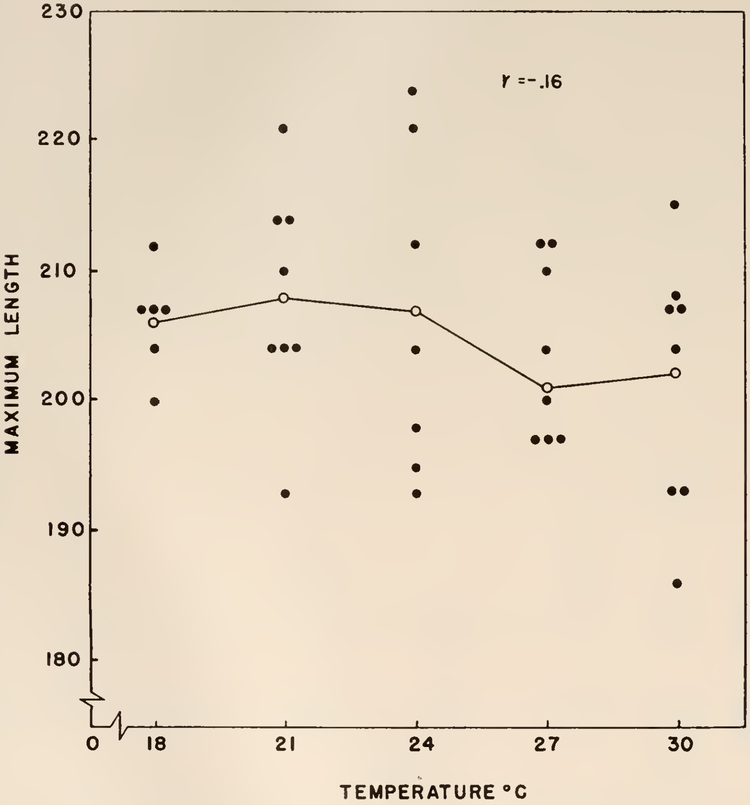

FIGURE 5. Maximum length of clam larvae of different cultures during the life of a culture, in relation to temperature. Measurements in microns. ● = mean of individual culture; o = mean for all cultures grown at the same temperature.

Although our studies showed that even if larvae are grown at different temperatures, they, in all cases, reach approximately the same mean length before setting, the question still unanswered is whether there is an appreciable relationship between the *maximum* length of larvae on the date of setting and the temperature. A statistical analysis demonstrated the lack of an appreciable relationship between these two variables, giving a correlation of − .16. The lack of relationship is clearly indicated in Figure 5, which shows that the means of the cultures grown at five different temperatures varied from 201 μ to 208 μ, a range of only seven microns. Nevertheless, we again noticed a considerable variability among the cultures within each temperature group, although it was much less pronounced within the 18.0° C. group than in certain others. However, again, no definite trend in this respect was observed because the variability of the maximum length of the larvae in the different cultures grown at 27.0° C., the second highest temperature group, did not differ greatly from that recorded for the lowest, *i.e.*, the 18.0° C. group.

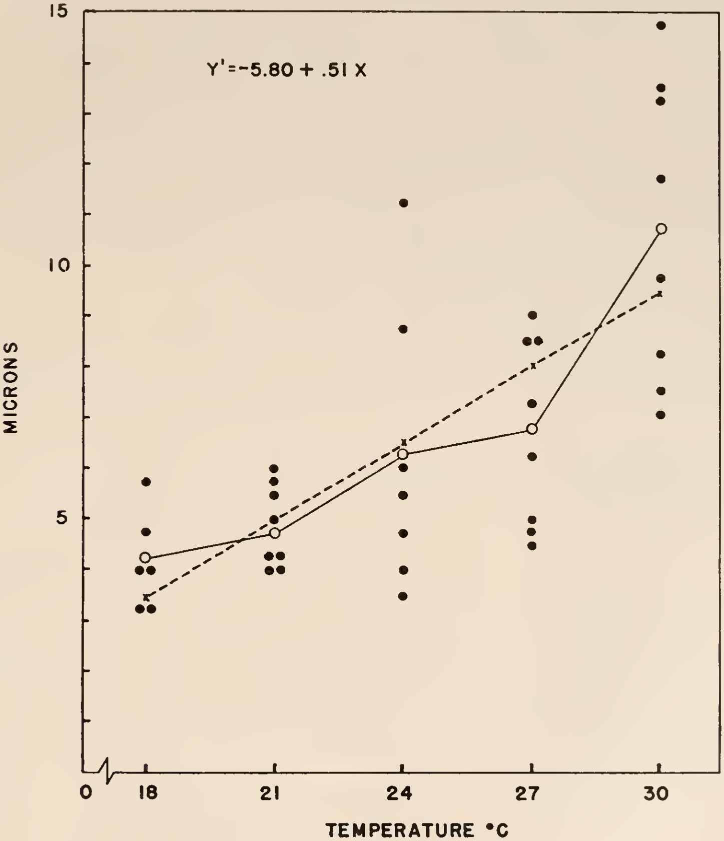

FIGURE 6. Average daily length increment of larvae grown at different temperatures. Measurements in microns. ● = mean of individual culture; o = mean of all cultures grown at the same temperature; x = mean for given temperature predicted from regression line.

Using the data collected during these studies, we can calculate the average daily growth increment for all cultures at given temperatures. By plotting the average daily growth increment for each temperature group against the corresponding temperature, an approximately rectilinear relationship becomes evident (Fig. 6). The regression equation computed was found to be $Y' = -5.80 + .51\,X$, where Y' is the predicted average daily growth increment and X is the temperature. The relationship indicates, naturally, that faster growth occurred at higher temperatures and, theoretically, from the regression equation it can be assumed that if the temperature were reduced to about 11.3° C., growth would stop completely.

In discussing our results it must be remembered that they are representative only for this series of experiments and that there are many factors which can change the daily growth increment. One of these, capable of lowering or increasing the rate of growth of larvae, is the quality of the food. In our experiments the larvae were fed a mixture of phytoplankton, consisting largely of *Chlorella.* However, recent studies of Davis and Guillard (1958) clearly showed that the growth of clam larvae varied greatly depending upon the kind of food they were given. Davis found that the larvae grew best when fed a culture of mixed flagellates, including *Isochrysis, Monochrysis, Dunaliella,* and *Platymonas,* while the larvae given *Chlorella,* two species of which were tried, grew considerably slower. In the same series of experiments Davis was able to demonstrate that the rate of growth of larvae of the American oyster, *Crassostrea virginica,* also varied greatly according to the kind of food organisms available.

Quantity of food organisms is another factor to be considered. Our earlier work (Loosanoff and Engle, 1947; Loosanoff, Davis and Chanley, 1953a) showed that heavy concentrations of food cells, such as *Chlorella,* usually seriously interfered with the feeding of adult oysters and that they also either killed the clam larvae or retarded their growth. Furthermore, they indicated that the optimum concentration of food organisms depended upon the kind and size of their cells. Since, in our experiments described in this article, the number of cells was not accurately determined and the food did not consist of pure cultures but of a mixture of many organisms, we do not know whether the clam larvae were fed the optimum food concentrations. This circumstance, however, does not invalidate our comparisons because all cultures were given food of the same quality and in the same quantity.

Finally, the effect of the concentration of larvae in the experimental cultures should be considered. Our studies (Loosanoff, Davis and Chanley, 1953b; Loosanoff, 1954) have shown that larvae in crowded cultures grow somewhat slower. However, the difference in the rate of growth of larvae in lightly-populated and those in densely overcrowded cultures was not too great. For example, we determined, at the end of the tenth day, that the mean length of larvae in the cultures containing only six individuals per cubic centimeter of water was 162 μ, whereas the mean length in the overcrowded cultures containing 52 individuals per cubic centimeter was 144 μ, or only 18 μ less than that recorded for lightly-populated cultures. Since, in the experiments described here, we began with the same number of larvae in all containers and because during the experiments no excessive mortality was recorded in any of the cultures, our larval populations in all jars were not much different from each other and, therefore, could not seriously affect the uniformity of the experimental conditions.

In concluding this article a brief reference to one more aspect of the role of water temperature on growth of bivalve larvae may be appropriate. It has frequently been reported that species living in warmer water have just as long a pelagic life as their northern relatives, and that, at a given temperature, the eggs of the southern species cleave and develop more slowly than those of the northern species of the same genus (Fox, 1936; Thorson, 1950). This suggests that even if the eggs and larvae were cultured under identical conditions, development of the eggs and larvae of the southern clam, *Venus* (*Mercenaria*) *campechiensis,* would require a longer period than is needed for eggs and larvae of the northern clam, *Venus* (*Mercenaria*) *mercenaria.* I had the opportunity to verify this contention by the studies conducted together with my associate, H. C. Davis. Adult *Venus* (*Mercenaria*) *campechiensis* were imported from the Apalachicola area of the Gulf of Mexico in November, 1953. Several weeks later these clams were conditioned for spawning. A group of large *Venus* (*Mercenaria*) *mercenaria,* natives of Long Island Sound, were ripened under identical conditions simultaneously with the southern species. When both groups were ripe, spawning was induced by our usual methods (Loosanoff and Davis, 1950). Fertilized eggs of each species and, later, larvae developing from these eggs were cultured under identical conditions, the temperature being approximately 21.0° C. Triplicate cultures of each species were grown, and random samples of 100 larvae from each culture were measured every second day. The curves constructed on the basis of this information showed that the rates of growth of the larvae of the two species were practically identical. Moreover, setting of larvae of both groups began at the same time. The results of this experiment contradict, therefore, the conclusion that when grown at the same temperature the eggs and larvae of the southern species develop more slowly than those of the northern species of the same genus.

I wish to express my thanks to Mrs. Barbara Myers for the statistical analysis of the data and to my associates, Miss Rita S. Riccio and Harry C. Davis, for their help in preparation of this article.

SUMMARY

1. The mean setting dates for larvae of *Venus* (*Mercenaria*) *mercenaria* grown at constant temperatures of 30.0°, 27.0°, 24.0°, 21.0° and 18.0° C. were found to lie on an almost perfectly straight line according to equation $Y' = -1.00\,X + 37.91$, where Y' is the predicted setting date and X is the temperature.

2. There were no significant differences among the five temperature groups with respect to mean length of larvae at time of setting.

3. There was no apparent relationship between *maximum* length of larvae at time of setting and temperature.

4. The correlation between mean width and mean length of larvae at time of setting was very high ($r = .95$).

5. No apparent relationship was found between shape of larvae (*i.e.,* ratio of mean length to mean width) at time of setting and temperature.

6. The average daily growth increment for all cultures at given temperatures under the conditions prevailing during the experiments was determined.

7. The rate of growth of larvae of the southern clam, *Venus* (*Mercenaria*) *campechiensis,* was the same as that of the northern species, *Venus* (*Mercenaria*) *mercenaria,* when the temperature and other conditions were identical. Moreover, setting of larvae of both species began at the same time.

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ANTIGENIC DIFFERENCES BETWEEN STEM AND HYDRANTH IN TUBULARIA[1]

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The marine hydroid, *Tubularia crocea,* consists essentially of two layers of tissue which are differentiated into a hydranth with hypostome, two groups of tentacles and gonophores, and a stem portion surrounded by a chitinous perisarc. Chemical differences may underlie these morphological differences between the hydranth and stem regions. Since the morphallactic regeneration of a hydranth from the stem region involves a remodeling of a certain portion of the stem tissues, further chemical changes may accompany visible morphological changes during regeneration of a hydranth. Because proteins and other complex molecules are the principal substances of organic forms, basic changes in morphogenesis probably involve production and transformation of such molecules and their aggregates.

The key role presumably played by the metabolism of complex molecules and especially proteins has interested developmental biologists for some time. A number of workers have used immunological methods to study differentiation of such substances and to correlate changes in their composition with visible morphological differentiation [reviewed by Irwin (1949), Ebert (1955), Tyler (1955, 1957), Woerdeman (1955), Schechtman (1955), Nace (1955), and Brachet (1957)]. While there have been many immunochemical studies concerned with embryonic development, immunological methods have rarely been used in studying problems of regeneration although the usefulness of such methods in studies on regeneration has been mentioned by Woerdeman (1953). De Haan (1956) and Laufer (1957) have used immunological techniques to study muscle differentiation in the regenerating limb of axolotl larvae.

In spite of numerous investigations on regeneration in *Tubularia* there is little information on the chemistry of this organism. A rational prerequisite to the investigation of chemical changes during hydranth regeneration would seem to involve the determination of chemical differences between the two main regions, the hydranth and the stem. Accordingly, the present study was undertaken to determine the similarities and differences in the antigenic composition of *Tubularia crocea* hydranths and stems (*cf.* preliminary note by Morrill, 1958).

MATERIALS AND METHODS

A species of *Tubularia, T. crocea,* was collected from the St. John's River jetties in Florida, where the organism is abundant from October to June. Only colonies whose stems were relatively free of macroscopic epizooites were used. After their collection the animals were kept in jars of sea water for not more than 36 hours.

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[2] The major portion of this study was performed during tenure of a National Science Foundation Predoctoral Fellowship.

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Clean whole animals, stems, and hydranths were frozen in a dry-ice bath. Hydranths and stems were prepared by first cutting off the hydranths and then removing the distal 4 millimeters of the stem plus any basal region of the stem that had epizooites. Stems were frozen within 20 minutes after hydranth removal. Hydranths were frozen within 4 hours after they had been severed from the stems. Separation of hydranths and stems on a sexual basis proved to be impractical. The frozen tissues were lyophilized and stored at − 20° C.

Preparation of saline extracts for use as antigens

One-gram quantities of lyophilized tissue were ground in a mortar and extracted with 10 ml. of buffered saline (9 g. NaCl/l., 0.1 M phosphate buffer, pH 7.0) for 8 to 12 hours at 4 to 10° C. At the end of this period the supension was homogenized in a glass homogenizer in an ice bath. The homogenate was kept at 4 to 10° C. for two more hours. Then the homogenate was centrifuged for 30 minutes at a centrifugal pressure of approximately 4500 g and the cloudy supernatant collected and stored at − 20° C. The nitrogen content of the extracts, determined by a modified nesselerization method (Hawk *et al.,* 1954), varied as follows: whole animal, 0.15 to 0.50 mg. N/ml.; hydranth, 0.25 to 0.82 mg. N/ml.; stem, 0.23 to 0.68 mg. N/ml.

The degree of contamination with insoluble particulate matter may have varied in the extracts. Therefore, equilibration on a nitrogen basis is at best an approximate equalization of antigen concentration.

Preparation of antisera

Nine male rabbits were used in the preparation of antisera. None of the pre-injection rabbit sera precipitated the extracts. Several injection routes were employed. These included intravenous, intermuscular with oil emulsion adjuvant (Freund, 1947), and intraperitoneal injections. The intraperitoneal route yielded the most satisfactory antisera. Two rabbits received three series of intraperitoneal injections over a two-month period. Each series consisted of 10 to 15 milliliters of whole animal extract administered within 6 to 8 days. The extracts injected into these rabbits were not adjusted on a nitrogen basis. One of these rabbits (Rabbit 4) was injected with the supernatant fraction of the homogenate. The other (Rabbit 6) was injected with the entire homogenate.

Blood was collected in sterile 50-milliliter centrifuge tubes 5, 7, and 17 days after the last injection of each series and allowed to clot at room temperature for 4 to 5 hours. The clots were loosened and the tubes stored at 4 to 10° C. for 24 hours. The serum then was decanted, centrifuged, and stored in vials at − 20° C. until used.

Serological tests

In order to test for antigenic differences between hydranth and stem tissues two types of precipitin tests were used. The first test employed was the standard interfacial ring test. When this test failed to reveal any antigenic differences, even when antiserum absorbed with hydranth or stem extract was used, the Ouchterlony agar gel diffusion method was employed in order to determine the spectrum of individual precipitin reactions and what differences might exist between the spectra of precipitin lines produced by anti-whole animal serum and stem and hydranth extracts.

Interfacial ring tests were performed by layering 0.05 ml. *Tubularia* extract over 0.05 ml. antiserum or over saline or pre-injection serum controls in 3 × 20 mm. capillary tubes. The tubes were capped with plastocene clay, incubated at 37° C., and examined after one and two hours for the presence of a precipitating ring at the interfaces. The highest antiserum titer (dilution of antiserum) of the sera from the two rabbits was 64. The highest antigen titer of the saline extracts was 16,000. These titers were obtained with the sera collected 5 and 7 days after the third injection series. No precipitates formed in control tests where pre-injection serum plus saline extracts and antiserum plus saline alone were employed.

The agar gel diffusion technique of Ouchterlony (Ouchterlony, 1949) was employed with modifications by Nace (personal communication). Details of the method are as follows. Four per cent agar was prepared and washed in distilled water for several days. This was used to prepare an aqueous 2 per cent agar containing aqueous merthiolate (0.25 ppth) and methyl orange (40 mg. per 500 ml. agar). A basal layer of this melted 2 per cent agar was poured on the bottom of petri dishes. After this layer had hardened additional agar was added and a lucite well mold placed in position. When the agar had hardened the mold was removed.

The wells were filled with antigens and antisera—0.30 ml. in the large square well and 0.15 ml. in the narrow rectangular wells. The center well of each plate was filled with antiserum or pre-injection serum and the four surrounding wells with saline extracts or saline. The plates were incubated in high humidity in an air-tight container at 37° C. for 7 days. They were then brought to room temperature for 6 to 24 hours and finally left at 5° C. for an additional 7 days.

At the end of 14 days the agar plates were fixed in 5 per cent formalin with methyl orange added, mounted between thin glass plates, and inserted into a photographic enlarger. The focused image of the wells and precipitin lines was so faint, particularly in the region of coalescence of lines, that photographing the preparations proved to be impractical. Therefore, the enlarged diagram of the lines was recorded by tracing on paper .

The antisera were absorbed in the following way: antisera and saline extracts or saline controls were mixed in various proportions in small sterile tubes, incubated at room temperature for two hours, placed in the refrigerator for 36 to 48 hours, and centrifuged. The supernatant was then subjected to interfacial ring tests and agar gel diffusion tests.

RESULTS

In order to test for differences in the antigenic composition of *Tubularia* hydranths and stems three tests were employed—the precipitin ring test, the Ouchterlony agar gel diffusion test, and the absorption test which was used in conjunction with the other two tests.

Precipitin ring tests

Precipitin antigen titers were determined in order to test for quantitative and qualitative differences between stem and hydranth extracts. Such extracts were equilibrated on a nitrogen basis, serially diluted and layered over anti-whole animal antiserum. No differences in the titers were observed. Evidently the two types of extracts were similar on a gross quantitative basis. To test the possibility of the existence of qualitative differences, absorption experiments were performed. Partial absorption of anti-whole animal antiserum by either extract produced antiserum which still reacted equally with both types of extract; complete absorption by either extract produced sera which failed to form a precipitin ring with either extract. The precipitin ring tests then failed to reveal any distinct antigenic differences between hydranth and stem extracts.

Because these tests failed to reveal any antigenic differences, it seemed desirable to utilize a method where the precipitate of the precipitin ring could be separated into a spectrum of one or more precipitin reactions. Accordingly, the antigenic composition of the extracts was examined by means of the agar gel diffusion technique of Ouchterlony.

Ouchterlony tests

Tests with unabsorbed sera. Preliminary Ouchterlony tests of anti-whole animal sera revealed multiplicity of precipitating systems. Similarities as well as differences existed between the precipitin patterns with stem and hydranth extracts. As expected, the number of lines formed varied with the antisera from the different rabbits. No precipitin lines appeared with pre-injection sera and saline controls. It was found that the pattern of precipitin lines varied in repeated tests with a given antiserum, possibly because the saline extracts used as test antigens were prepared at different times, even though the extracts were prepared by a standard procedure and adjusted on a nitrogen basis. In addition, antisera from a rabbit taken at different times following a series of injections exhibited variations in position and in number of lines in the precipitate patterns when tested with hydranth and stem extracts. This is probably because the maximum concentration of antibodies for the several antigens did not occur at the same time (Abramoff and Wolfe, 1956). The most complete precipitin line patterns were obtained with antisera from Rabbit 4 and Rabbit 6 (see under Methods) obtained 5 and 7 days after the third series of intraperitoneal injections.

The best precipitin pattern produced by anti-whole animal sera with hydranth and stem extracts is given in Figure 1. This antiserum from Rabbit 4 produced a total of seven precipitin lines with hydranth extract and seven precipitin lines with stem extract. Six precipitin lines with hydranth extract coalesced with five lines produced with stem extract. One additional line was restricted to the hydranth extract. Two precipitin lines and a spur on a coalescing line were restricted to the stem extract. Fewer lines were produced with antiserum of Rabbit 6. In the best pattern with antiserum from this rabbit four lines produced by hydranth extract coalesced with three lines formed by hydranth extract. In addition two precipitin lines were limited to reactions with components of stem extract, and possibly one was limited to hydranth extract.

In the several experiments the coalescing lines formed complex patterns. In addition to the fusion of single lines formed by the two extracts, there were instances where two hydranth precipitin lines coalesced with one line formed by stem extract and vice versa. These results may be interpreted as being due to superimposed precipitin lines in the reaction of the antiserum with one extract. It is also possible that in one extract an antigenic substance had haptens in common with the haptens of two antigenic substances in the other extract (Kaminski and Ouchterlony, 1951). In nearly all the experiments one of the stem lines that coalesced with a hydranth line had a spur which extended beyond the region of coalescence (Fig. 1). This indicates that this stem antigen had two haptens, one in common with a hydranth antigen and one not found on any of the hydranth antigens. This interpretation is in accord with the explanation for the appearance of spurs given by Kaminski and Ouchterlony (1951).

The large number of lines formed in the Ouchterlony patterns suggested that some of the lines might be due to excessive quantities of certain antigens or antibodies (Kaminski, 1954). It was also possible that one or more of the single lines might be due to several superimposed precipitates (Grasset *et al.,* 1956). Attempts to resolve the complexity of the patterns, however, by diluting either the antisera or the hydranth and stem extracts resulted in patterns with fewer and more diffuse lines.

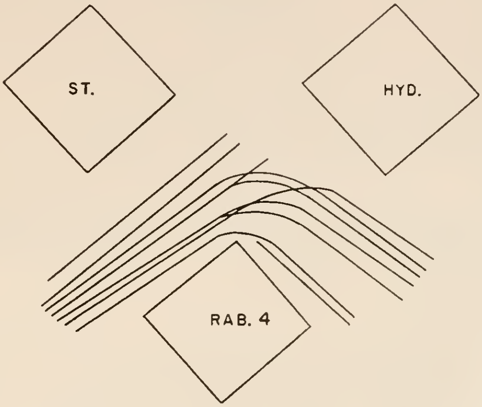

FIGURE 1. Diagram of precipitin pattern of anti-whole animal serum, Rabbit 4, with stem and hydranth saline extracts.

The number of lines was also checked by the immunoelectrophoretic method of Grabar and Williams (Grabar and Williams, 1953, 1955). In the first experiment the antigens of whole animal extract were separated in 2 per cent agar electrophoretically (veronal buffer, pH 8.6, ionic strength 0.1, 10 ma., 24 hrs.). After the separation, anti-whole animal serum of Rabbit 4 was placed in a trench parallel to the path of migration and allowed to diffuse into the agar. After seven days' incubation, nine precipitin lines in the form of distinct arcs were detected. In another experiment extracts of hydranths and stems were separated electrophoretically (veronal buffer, pH 8.6, ionic strength 0.05, 30 ma., 5 hrs.) and tested with anti-hydranth serum. This antiserum produced six curving precipitin lines with each extract. The electrophoretic mobilities of the several arcs were similar. This particular antiserum when previously tested by the Ouchterlony method had produced six lines with hydranth extract and five lines with stem extract.

The patterns formed by coalescing lines in the Ouchterlony tests demonstrate a number of antigens to be common to both hydranth and stem. The immunoelectrophoretic patterns showed that at least six of the hydranth antigens had electrophoretic mobilities comparable to those of six stem antigens. In addition to the similar antigens the tests with unabsorbed sera showed one antigen to be limited to the hydranth and at least two antigens limited to the stem.

Tests with absorbed anti-whole animal serum. In order to resolve further the antigenic differences between hydranth and stem extracts, absorbed serum (Rabbit 6) was used in Ouchterlony tests. Antiserum absorbed with hydranth extract still produced two distinct and one faint, diffuse precipitin lines with stem extracts. Antiserum absorbed with stem extract produced one precipitin line with hydranth extract. The absorbed sera failed to produce bands with the absorbing antigen. These results again indicate there are at least two antigenic substances restricted to stems and one restricted to hydranths.

Discussion

The results demonstrate that precipitating antibodies can be obtained against saline extracts of *Tubularia.* The agar diffusion tests show that a spectrum of precipitating antigens is present in stems and hydranths.

It is possible that some of these antigenic substances are not actually part of the tubularian tissues. In spite of the precautions described (see methods section), the antigens restricted to the stem extracts may be from organisms associated with the perisarc which is limited to the stem region. The antigenic differences may also be due to breakdown products of ingested food. The discussion presented here is subject to these reservations.

A number of antigens appear to be common to both hydranths and stems. It was previously reported (Morrill, 1958) that there were seven common antigens. Re-examination of Ouchterlony patterns has revealed that at least three antigens are common to both regions of the animal. In addition, one stem antigen has antigenic sites similar to those on two hydranth antigens. Immunoelectrophoretic experiments with anti-hydranth serum showed six antigens of hydranths and stems to have similar electrophoretic mobilities. This method resulted in distinct non-overlapping lines and should prove useful in future studies on the antigenic composition of this organism. Ouchterlony tests with non-absorbed and absorbed anti-whole animal sera indicate that at least two antigenic substances are limited to stems and one to hydranths.

With the establishment of antigenic relations between stems and mature hydranths further investigations need to be conducted to determine the antigenic relations between stems, regenerated hydranths, and hydranths at different stages of regeneration. The antigens need also to be characterized. Preliminary experiments show that antisera inhibit hydranth regeneration. Hydranth-specific and stem-specific antibodies should now be tested for inhibiting action on the regeneration of hydranths.

I wish to express my appreciation to Dr. Charles B. Metz for his guidance, encouragement, and interest during the course of this investigation.

SUMMARY

1. The antigenic composition of hydranths and stems of *Tubularia crocea* has been studied by means of the precipitin ring tests, Ouchterlony agar gel diffusion tests, and immunoelectrophoresis.

2. Precipitin ring tests showed that antiserum against whole animals contained precipitating antibodies but failed to reveal antigenic differences between hydranths and stems.

3. Ouchterlony tests of anti-whole animal serum and saline extracts of hydranth and stem tissues revealed the following:

a. Four antigens common to both regions of the animal.
b. One stem antigen with hapten sites similar to those on two hydranth antigens.
c. Two antigenic substances limited to stems.
d. One antigenic substance limited to hydranths.
e. One stem antigen with at least two haptens—one in common with a hydranth antigen and one which was not related to any precipitating hydranth antigens.

4. Six stem antigens and six hydranth antigens had comparable electrophoretic mobilities.

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ADENOSINETRIPHOSPHATASE OF MYTILUS SPERMATOZOA. II. EFFECTS OF SULFHYDRYL REAGENTS, TEMPERATURE AND INORGANIC PYROPHOSPHATE[1]

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The enzyme concentrated in the sperm flagellum of the mollusc, *Mytilus edulis,* which splits adenosinetriphosphate (ATP) has been classified as a "true" adenosinetriphosphatase (ATP-ase) (Nelson, 1955). Mohri (1958) has confirmed this in studies on the flagellar ATP-ase of sea urchin sperm. This class of enzyme liberates only one phosphate group from each ATP molecule, even on prolonged incubation. The Mytilus sperm enzyme exhibits optimum activity at about pH 8.4 in veronal and glycine buffers, while for sea urchin sperm, pH 8.8 is optimum in Tris and veronal buffers. Magnesium has a pronounced activating effect on both the molluscan and the echinoderm sperm ATP-ase; however, calcium exerts considerably less activation, and, moreover, antagonizes the potentiation due to magnesium. Dilution of filtered-sea-water or isotonic KCl suspensions of Asterias and Mytilus sperm tails with large volumes of glass-distilled water or 10^{-4} *M* $MgCl_2$ causes slow precipitation of the tails; this may be accelerated by the addition of small amounts of ATP. However, extruded threads produced from these sperm tail suspensions do not contract; this may be attributed to the lack of continuity of the components—the individual sperm tails (unpublished observation). Salts of heavy metals and other sulfhydryl reagents which serve as spermicidal agents act to halt sperm motility and also interfere with a regulatory mechanism of sperm respiration; low concentrations of inhibitor permit "uncontrolled" acceleration of oxygen consumption, while higher concentrations completely stop O_2 uptake and motility (Barron *et al.*, 1948; MacLeod, 1951). It has also been observed that sodium pyrophosphate, apparently by forming firm complexes with the divalent cations essential for optimal activity, exerts an inhibitory action on sperm ATP-ase (unpublished). The first paper in this series dealt with some of the characteristics of the Mytilus sperm tail ATP-ase. The effect of some additional agents (SH-inhibitors, temperature, pyrophosphate) on spermatozoan ATP-ase is considered in the investigations reported here.

MATERIALS AND METHODS

Since it is difficult to induce spawning in the Woods Hole Mytilus either by temperature shock or the injection of KCl, it was necessary to obtain the sperm from minces of the gonads. The sperm were harvested and prepared as reported previously (Nelson, 1955). "Decapitation" was effected by means of a stainless steel, piston-type homogenizer, and the heads and tails separated by repeated gentle centrifugation (1000 × g for ten minutes each) in isotonic KCl. The sedimented heads were discarded. The dilution-precipitation effect was exploited in the further isolation and purification of the tails from the pooled supernatant fractions by the addition of at least five volumes of ice-cold 10^{-4} M $MgCl_2$. After standing in the refrigerator for 6 to 12 hours, the tails had settled out and the clear supernate was decanted and discarded. The flocculent precipitate was further concentrated by centrifugation at 7000 × g for 10 minutes. The sperm tail concentrate was then taken up in two to four volumes of isotonic ($M/2$) KCl. (All solutions were made up in de-ionized water.) Since the tail preparation resisted solubilization in mild alkaline and detergent solutions, no further effort was made to extract them and the experiments were performed on the "intact" tails. The reactants, mixed by lateral agitation in 12-ml. Pyrex conical centrifuge tubes, consisted of 0.1 or 0.2 ml. of sperm tail suspension, 0.8 or 0.7 ml. Tris buffer (Sigma), pH 8.6 (0.05 M KCl, 10^{-4} M $MgCl_2$, 0.04 M Tris), unless otherwise noted. After the reactants had equilibrated in a thermostat at 24° to 25° C., 0.1 ml. 10^{-3} M ATP (Sigma disodium salt, neutralized with NaOH to bromthymol blue endpoint) was added and the mixture allowed to incubate for ten minutes. Addition of one ml. of ice-cold 10% trichloroacetic acid terminated the reaction. The precipitate was

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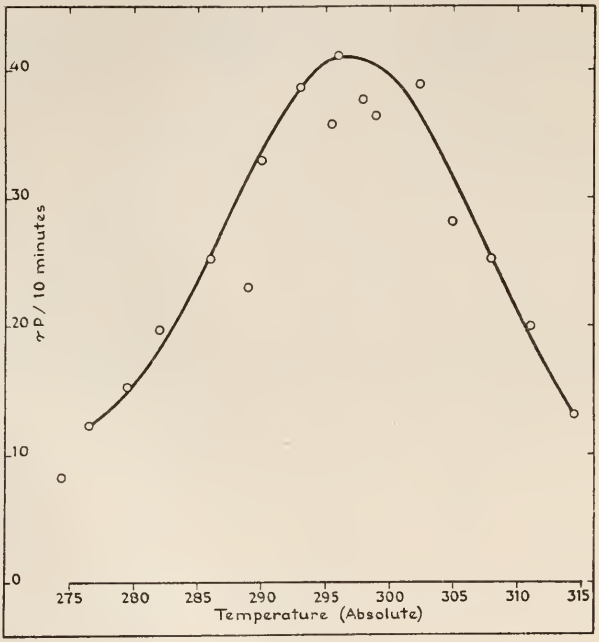

FIGURE 1. Temperature dependence of Mytilus sperm tail ATP-ase. Reaction mixture: 0.05 M KCl; 0.04 M Tris buffer, pH 8.6; 10^{-4} M $MgCl_2$; 10^{-3} M ATP; 0.1 ml. sperm tail suspension; total volume: 1.0 ml. Incubation time, 10 minutes. Ordinate, phosphate liberated in 10 minutes; abscissa, temperature, degrees, Absolute.

removed by centrifugation and the entire supernate analyzed for orthophosphate by the microcolorimetric method of Taussky and Shorr (1953). Optical density was measured in a Coleman, Junior spectrophotometer at a wave-length of 660 millimicrons. Protein content of the sperm tail samples was estimated by a modification of the method described by Nielsen (1958).

RESULTS

Temperature-dependence of flagellar ATP-ase. Duplicate determinations of the enzyme activity at various temperatures over a range from 1° to 41.5° C. show a fairly constant increase in rate up to about 20° C., virtually a doubling for each ten-degree rise ($Q_{10} = 2$) (Figure 1). Between 20° and 30°, the rate of dephosphorylation levels off and then declines fairly uniformly. From the slope of the Arrhenius plot (Fig. 2), the activation energy of the reaction was calculated to be − 10,450 cal./degree/mole. Since these determinations have been made on crude preparations, on "whole" tail suspensions, rather than purified enzyme extracts, this finding suggests that the broad temperature range of maximum enzyme action may simulate the situation which occurs during natural spawning. This coincidence may be of significance in that when associated with other factors (chemotactic, antigenic, etc.; *cf.* review by Rothschild, 1956) which may operate to assure maximum fertilization, optimum swimming activity of the spermatozoa may further serve

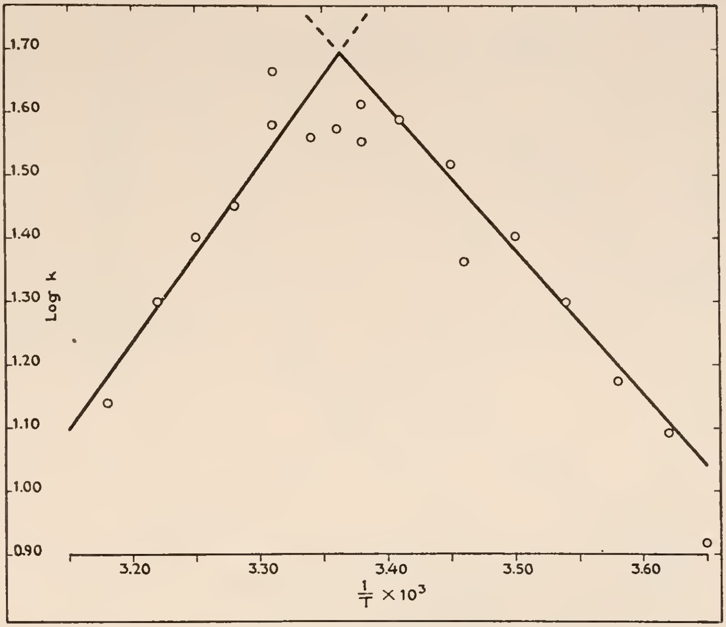

FIGURE 2. Temperature dependence of Mytilus sperm tail ATP-ase. Arrhenius plot of data in Figure 1. Ordinate, logarithm of rate of phosphate liberated; abscissa, reciprocal of temperature, 1/degrees, Absolute.

to increase the number of effective sperm "collisions" with activatable eggs. The dependence of spermatozoan motility on utilization of ATP has been established (Mann, 1945; Rothschild and Mann, 1950; Nelson, 1958a).

Effect of sodium pyrophosphate on sperm ATP-ase

Presence of an inorganic pyrophosphatase in flagella. Preliminary observations indicated that when the sperm tail incubation medium contained ATP and sodium pyrophosphate (NaPP) in the ratio of 1.6:1, the amount of inorganic phosphate liberated was 88% of the control (no NaPP). When the molar ratio of ATP to NaPP was 0.8:1, only 57% of the control activity was found. Tentatively, this was interpreted as an inhibition caused by the removal of the activating divalent cations through their chelation by the pyrophosphate. Verification was deferred until the present.

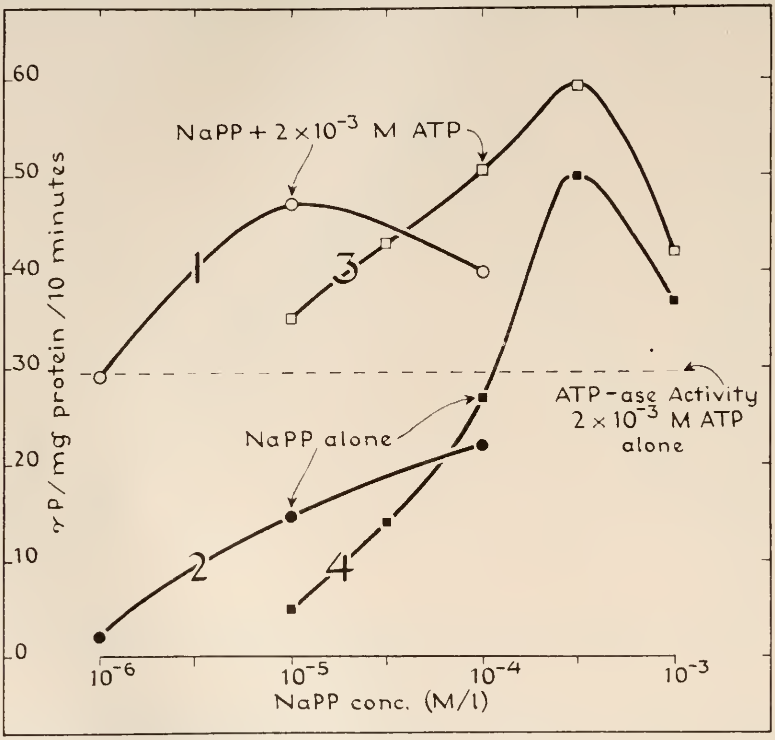

FIGURE 3. ATP-ase and IPP-ase activity of isolated Mytilus sperm tails. Reaction mixture: 0.05 *M* KCl; 0.04 Tris buffer, pH 8.6; 5×10^{-5} *M* $MgCl_2$ (dotted line and Curve 1, open circles, and Curve 2, closed circles) or 10^{-4} *M* $MgCl_2$ (Curve 3, open squares and Curve 4, closed squares); 2×10^{-3} *M* ATP (Curves 1 and 3 and dotted line); varying concentrations of NaPP; 0.1 ml. sperm tail suspension. Total volume, 1.0 ml. Incubation: 10 minutes, 24.5° C. Ordinate, γ phosphate liberated/mg. protein/10 minutes, abscissa NaPP concentration (M/liter).

The sperm tail suspension, incubated for 10 minutes at 24.5° C. in 2×10^{-3} *M*

ATP and 5×10^{-5} *M* $MgCl_2$, splits off about 30 γ phosphate per mg. of sperm tail protein (dashed line, Fig. 3). When varying amounts of NaPP are added to this mixture, the increase in phosphate liberation evidently depends on the relative concentrations of NaPP and $MgCl_2$ in the medium. Curve 1 (open circles) shows that a peak of activity is reached at 10^{-5} *M* NaPP in the ATP-containing medium; while at a somewhat higher $MgCl_2$ (10^{-4} *M*) concentration, curves 3 and 4 (open and closed squares) exhibit peaks of activity at 5×10^{-4} *M* NaPP, both in the presence and absence of ATP.

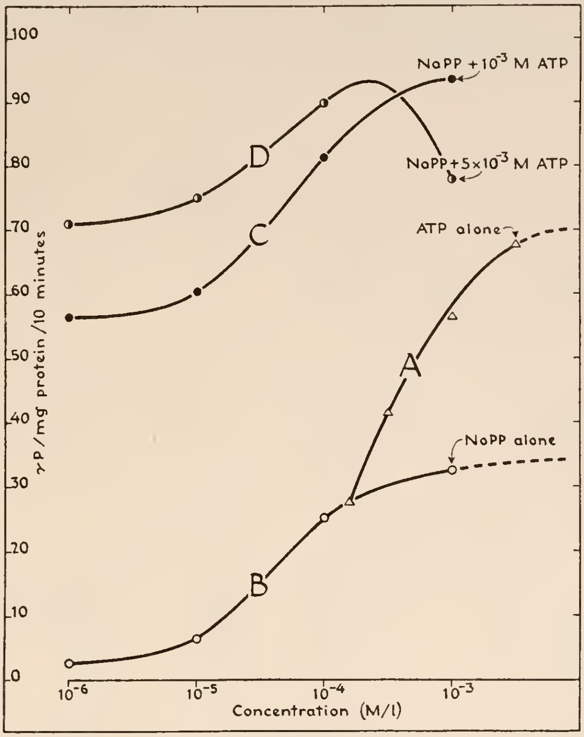

FIGURE 4. ATP-ase and IPP-ase activity of isolated Mytilus sperm tails. Reaction mixture: 0.05 *M* KCl; 0.04 *M* Tris buffer, pH 8.6; 5×10^{-5} *M* $MgCl_2$; varying concentration of NaPP (Curves B, C, D, open, closed and half circles, respectively); varying concentrations of ATP (Curve A, open triangles); 10^{-3} *M* ATP (Curve C), 5×10^{-3} *M* ATP (Curve D); 0.1 ml. sperm tail suspension (more concentrated than in previous figures). Total volume 1.0 ml. Incubation: 10 minutes, 24° C. Ordinate, γ phosphate liberated/mg. protein/10 minutes. Abscissa, concentration of phosphate ester (NaPP—curves B, C, D; ATP—curve A).

It is apparent from these results that (i) the sperm tail preparations exhibit an active inorganic pyrophosphatase (IPP-ase); (ii) that this enzyme activity is additive to that of the ATP-ase; (iii) that above an optimum substrate (NaPP) concentration there is a depression of the IPP-ase; (iv) that this optimum may be related to the magnesium concentration; and (v) that the presence of ATP may even aggravate the depression of IPP-ase activity under certain circumstances.

These observations were confirmed and the situation elucidated with a fresh sperm tail preparation. The conditions of the experiment were adjusted by doubling the $MgCl_2$ concentration (from 5×10^{-5} *M* to 10^{-4} *M* $MgCl_2$), by making up the ATP in the Tris buffer-KCl-$MgCl_2$ medium to maintain the total [$MgCl_2$] constant while varying the [ATP] and by increasing the sperm tail content in the incubation mixture (Fig. 4). The ATP-ase activity curve (A) approaches a maximum velocity of 70 γ phosphate/mg. protein/10 min. with increasing substrate concentration. The IPP-ase activity approaches a maximum velocity about half that of the ATP-ase (34.5 γ phosphate/mg./protein/10 min., Curve B). When, as shown in Curve C, increasing amounts of NaPP are added to the sperm tail incubation medium containing 10^{-3} *M* ATP, the activity is increased by 50 to 60 γ phosphate at all concentrations of NaPP, so that activity curves B and C appear parallel. However, when the medium contains 5×10^{-3} *M* ATP plus increments of NaPP (Curve D), the initial increase in rate of dephosphorylation approximates that attributable to the increase in ATP concentrations. Subsequently the rate increases slowly, approaching the maximum attained in Curve C, although at about ⅕ the NaPP concentration. Thereafter the velocity of the enzymic action declines rapidly.

Table I

1 Substrate concentration (M/liter)			2 Phosphate liberated (γ)			3 Difference	
ATP	NaPP	ATP + NaPP	Observed or extrapolated	If ATP	If NaPP	γ Phosphate	
			a	b	c	a − b	a − c
0.001			56	56	32.5	0	23.5
	0.000001		2.5				
		0.001001	56	56.5	32.5	−0.5	23.5
	0.00001		6.5				
		0.00101	60	57	32.5	3	27.5
	0.0001		25				
		0.0011	81	60	33	21	48
	0.001		32.5				
		0.002	93.5	62	33.2	31.5	60.3
0.005			67.5	67.5	34.5*	0	33
	0.005001		71	67.5	34.5*	3.5	36.5
	0.00501		75	67.5	34.5*	7.5	41.5
	0.0051		90	68	34.5*	22	55.5
	0.006		78	69*	34.5*	9	43.5

* V_{max} calculated from Lineweaver-Burk plot (Fig. 5).

ATP-ase and IPP-ase activity. Reaction mixture: 0.05 *M* KCl, 0.04 *M* Tris, pH 8.6, 5×10^{-5} *M* $MgCl_2$; 0.1 ml. sperm suspension; and varying concentrations of ATP and NaPP. Total volume, 1.0 ml. Incubation conditions: 10 minutes, 24.5° C. Enzyme activity = γ phosphate split/mg. protein/10 minutes incubation.

The data summarized in Table I support the conclusion that two distinct enzymes are involved in the dephosphorylation of ATP and NaPP. If all the hydrolyzable phosphate were present as ATP, in Curve C of Figure 4, the maximum amount of phosphate split under the conditions of this experiment (enzyme concentration limiting) could not greatly exceed 62 γ instead of 93.5 γ (column 2); if the total source of P were NaPP, then the maximum P liberated probably could be only 33.2 γ. Similarly, if total hydrolyzable P in Curve D were present in the form of ATP, the maximum liberated P would not exceed 69 γ, or if present in the form of NaPP, probably would not be in excess of 34.5 γ. It is further evident that when both sperm tail content and $MgCl_2$ concentration in the incubation mixture are increased, in contrast to the situation in Figure 2, the NaPP does not interfere with itself as suggested by the optimum in curve 4, Figure 2, but that addition of

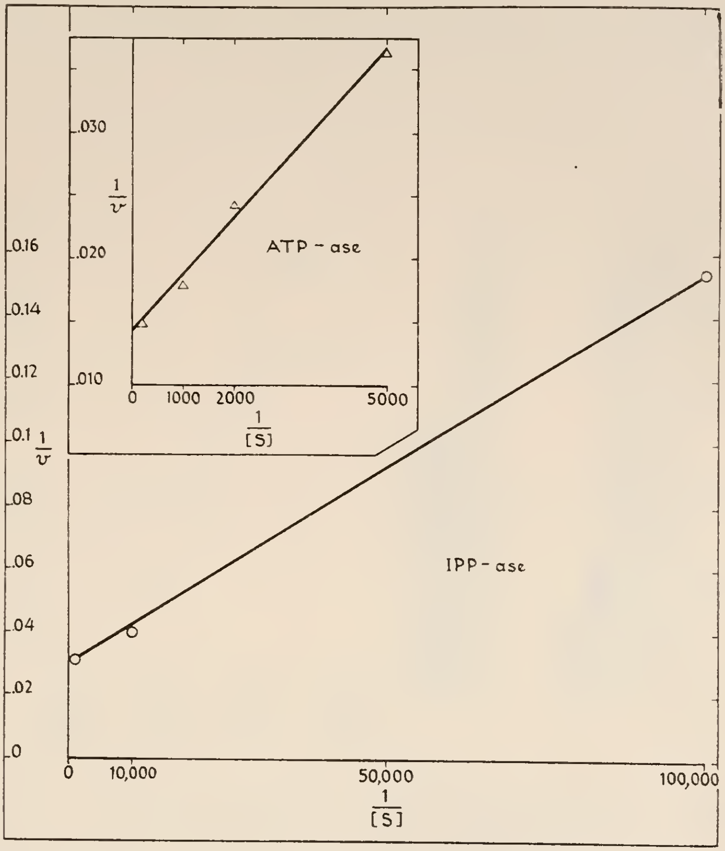

FIGURE 5. ATP-ase and IPP-ase activity of isolated Mytilus sperm tails. Lineweaver-Burk plot. Reaction mixture: 0.05 *M* KCl; 0.04 *M* Tris buffer, pH 8.6; 5×10^{-5} *M* $MgCl_2$, 0.1 ml. sperm tail suspension; varying concentrations of substrate; total 1.0 ml. Incubation: 10 minutes, 24° C. Open circles—IPP-ase; inset-open triangles—ATP-ase. Ordinate, reciprocal rate of phosphate liberation/mg. protein/10 minutes; abscissa, reciprocal of substrate concentration. $K_{M\text{-IPP-ase}} = 4.3 \times 10^{-5}$; $V_{max\text{-IPP-ase}} = 34.5$. $K_{M\text{-ATP-ase}} = 3.1 \times 10^{-4}$; $V_{max\text{-ATP-ase}} = 70$.

ATP at high enough concentration duplicates this phenomenon (Curve D, Fig. 3). Mohri (1958) and Nelson (1955) have shown the distinctive magnesium activation of the sperm tail ATP-ase. Evidence from the literature suggests that, with one notable exception cited in Discussion, regardless of enzyme source, inorganic pyrophosphatase activity is generally limited by the magnesium content of the medium. In this respect, the flagellar IPP-ase is not unique. The mutual dependence of these enzymes on adequate magnesium ion suggests the validity of the original concept that by chelation, NaPP competes with ATP for the magnesium ions and thereby could exert an inhibitory influence on the ATP-ase activity. The possible significance for sperm motility of this mutual interaction of substrates and enzyme activities will be considered later.

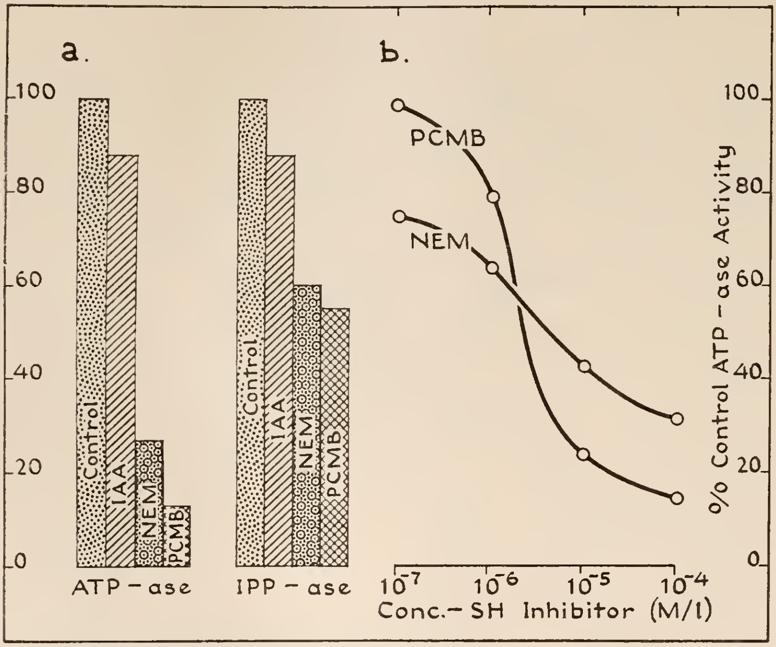

FIGURE 6. Effect of sulfhydryl inhibitors on Mytilus sperm tail enzymes. a. Effect of 10^{-4} *M* SH-inhibitor: Ordinate, per cent of control enzyme activity; abscissa, bar 1, control; bar 2, monoiodoacetate; bar 3, N-ethyl maleimide; bar 4, p-chloromercuribenzoate. b. Curves showing effect of varying concentrations of N-ethyl maleimide and p-chloromercuribenzoate on sperm tail ATP-ase. Ordinate, per cent of control ATP-ase (no inhibitor); abscissa, concentration of SH-inhibitor (*M*/liter). Incubation: 10 minutes, 24.5° C.

When the Lineweaver-Burk (1934) analysis is applied to these unpurified preparations, the Michaelis constants K_m, and the maximum velocities V_{max} of the respective enzymatic reactions may be determined graphically (Fig. 5). For the IPP-ase, K_m is 4.3×10^{-5}, and V_{max} is 34.5γ P/mg. protein/10 min., while for ATP-ase K_m is 3.1×10^{-4}, and V_{max} is 70γ P/mg. protein/10 min. (K_m for erythrocyte IPP-ase equals 5.4×10^{-4} according to Bloch-Frankenthal, 1954; and K_m for skeletal muscle ATP-ase equals 1×10^{-4} to 3×10^{-4}, according to Watanabe *et al.*, 1952.) Under these conditions, Q_p of the ATP-ase equals 300 as compared to 150 for the bull sperm ATP-ase (Nelson, 1954), and would probably be somewhat higher for purified sperm tail extracts. Further evidence substantiating the belief that two separate enzymes are involved derives from comparison of the effects of sulfhydryl reagents on the dephosphorylation of the two substrates.

Sulfhydryl inhibition of flagellar ATP-ase and IPP-ase. The different sulfhydryl inhibitors caused varying degrees of inhibition of the enzymes. ATP-ase is more sensitive than IPP-ase to the action of both N-ethyl maleimide and p-chloromercuribenzoate at an inhibitor concentration of 10^{-4} *M*, while sodium monoiodacetate inhibits both enzymes only very slightly (bar graphs, Fig. 6a). ($HgCl_2$ at the same concentration completely inhibits the ATP-ase.) P-chloromercuribenzoate, a mercaptide-forming agent, is more effective at higher concentrations, while N-ethyl maleimide, an alkylating agent, is relatively more potent at lower concentrations in inhibiting the sperm tail ATP-ase (Fig. 6b). This may reflect the fact that while N-ethyl maleimide is a specific sulfhydryl inhibitor, p-chloromercuribenzoate at the higher concentrations may be combining with other reactive and essential sites on the protein side-chains in addition to sulfhydryls (Boyer, 1959). Unfortunately, no attempt was made at the time to reactivate the enzymes by treatment with sulfhydryl compounds such as BAL (2, 3 dimercaptopropanol), glutathione or cysteine.

Discussion

The studies on the temperature dependence and the effects of sulfhydryl inhibitors are relatively straightforward. Kielley and Bradley (1956) reported that with calcium as activator, when approximately one-half of the sulfhydryl groups of myosin are titrated with either p-chloromercuribenzoate or N-ethyl maleimide a marked increase in ATP-ase activity occurs. Other distinguishing features of the myosin- and actomyosin-ATP-ase and sperm tail ATP-ase have already been considered (Nelson, 1955). Under the conditions of the present experiments, the flagella again differ from the muscle ATP-ases in exhibiting none of the sulfhydryl reagent activation; whether this is characteristic only of the purified enzyme or of Ca-activated ATP-ase, remains to be investigated.

Of particular interest has been the finding that the sperm tails actively dephosphorylate pyrophosphate. Mohri (1958) concludes that Hemicentrotus sperm tails which hydrolyze ATP are enzymatically inactive to a number of other phosphate esters among which he includes inorganic pyrophosphate. Heppel and Hilmoe (1951) describe an inorganic pyrophosphatase in bull seminal plasma, with a sharp optimum at pH 8.6. The bull seminal IPP-ase has nearly maximal activity in the absence of magnesium, while firefly (McElroy *et al.*, 1951, 1953) and yeast IPP-ase have an absolute requirement for Mg^{++}, as apparently does that of Mytilus sperm tail (although this requires further study). The bull seminal IPP-ase, unlike that of yeast and the sperm flagellum, shows no inhibition by increased substrate. Since metaphosphate, which also forms firm complexes with Mg^{++}, but is not acted on by the enzyme, inhibits yeast IPP-ase in the same concentration range as pyrophosphate, Heppel and Hilmoe (*loc. cit.*) attribute inhibition by high pyrophosphate to Mg^{++} binding. This interpretation is substantiated by the present studies involving the combined action of ATP and NaPP. Moreover, inorganic pyrophosphate inhibits magnesium-activated myofibrillar ATP-ase when the total concentration of ATP and NaPP exceeds that of the $MgCl_2$ (Perry and Grey, 1956), and decreases the light scattering of actomyosin solution in the presence of magnesium (Tonomura *et al.,* 1952) even though the pyrophosphate is not split. A number of the nucleoside triphosphates also possess this property of modifying, or interfering with the myosin or actomyosin interaction with ATP, and so the behavior of inorganic pyrophosphate is not unique in this respect (Hasselbach, 1956). However, when considered in conjunction with the activity of the enzyme inorganic pyrophosphatase, this substance assumes peculiar significance and invites speculation as to its possible role as a regulator of a specific cellular energetic reaction. To cite several instances, in addition to vertebrate muscle, for which inorganic pyrophosphate may also serve as an extractant, both the substrate and enzyme may be involved in such diverse activities as firefly luminescence and insect flight. McElroy and his co-workers (1953) report that the decrease in light intensity after mixing ATP, luciferin, luciferase, O_2, and Mg^{++} is due to the formation of an inactive complex of luciferase which depends on magnesium and a second protein, namely, IPP-ase. Addition of pyrophosphate causes a sharp increase in the light, but inhibitors of the pyrophosphatase (Mn^{++}, Ca^{++}, F^-) must be added to prevent the rapid decay of the high light intensity obtained with the pyrophosphate. (However, iodoacetate, even at concentrations of 10^{-3} and 10^{-2} *M* does not inhibit this pyrophosphatase.) Gilmour and Calaby (1953) suggest the possibility that pyrophosphate hydrolysis may have some importance as a source of energy for cellular processes, since locust thoracic muscle pyrophosphatase is three times higher than that of femoral muscle, and also refer to the report that the heat of hydrolysis of pyrophosphate is approximately 9000 cal./mole (Ohlmeyer and Shatas, 1952). It is unlikely that such an enzymatic reaction is without physiological consequence. An interpretation in harmony with the wide variety of evidences of pyrophosphate involvement in cellular processes may be deduced from evidence that pyrophosphate is one of the naturally occurring "relaxing" or plasticizing factors. Pyrophosphate duplicates the softening effect of ATP in glycerinated muscle fibers and sperm flagella, so-called "cell models" (Bishop, 1958a). Magnesium is essential for the production and maintenance of the extensibility and plasticity of glycerol-extracted muscle (Bozler, 1954a), by physiological concentrations both of ATP and of NaPP. Bozler (1954b) proposed that relaxation is caused by the inactivation of bound calcium and that the relaxed state is due to the formation of an enzymatically inactive protein-ATP-Mg complex. He suggests (p. 157) that "the effect of ATP depends on a balance between two antagonistic actions, contraction, which is caused by the breakdown of ATP, and a softening action like that caused by PP. Whether contraction or relaxation occurs then depends on which of these effects predominates."

["Elementary processes in muscle action," Morales *et al.* (1955) should be consulted for a review of the actions of two other naturally occurring modifying factors, myokinase and the system ATP-creatine-transphosphorylase + creatine phosphate, as well as EDTA, and the features held in common by these very different substances.] Bishop (1958b) believes that one or more of the relaxing systems may play roles in sperm model "motility," since glycerinated sperm twitch repetitively on addition of ATP, ADP, or ITP, while pyrophosphate increases the amplitude of the twitch induced by these substances in rodent sperm. However, these models are capable of very little, if any, progressive movement, the rate of oscillation is usually slower than that of fresh sperm, the wave is not propagated, and the movements are occasionally restricted to one or another portion of the tail. In this connection, it is worth noting that inorganic pyrophosphatase is a water-soluble enzyme, and as such is one of several components extracted upon glycerination (Nelson, 1959). Isolated fresh sperm tails, also, may oscillate or twitch (Gray, 1958), and so the control or regulation of the undulatory flagellation is most likely an autonomous function of the flagellum itself. Initiation of motile activity may depend on "extraneous" excitatory factors, *e.g.*, the so-called dilution effect, hormones, partial pressure of O_2, or CO_2, etc. (*cf.* Mann, 1954). But once the sperm is activated, propagated contraction waves progress down each of the nine outer longitudinal fibers in sequence in such a fashion that while one fiber is contracting, the ones opposite are plastic, undergoing relaxation-activation cycles which immediately succeed their own contraction waves (Nelson, 1958b). This sequence could impart the spiral twist observed in the undulatory wave and perhaps as well the spiral thrust which characterizes the progressive movement of the sperm. In cytochemical terms this may be visualized as follows: The contractile protein-Mg-ATP complex is the condition of the "active" state. Upon contraction, a rigor-like state might ensue, except that pyrophosphate then combines with the contracted fiber through Mg^{++}, inducing the relaxation phase; but since NaPP cannot replace the contraction-inducing property of ATP, and the kinetics of dephosphorylation suggest that the two substances are mutually inhibitory at physiological levels, the NaPP "block" must be removed from the spatial proximity of the contractile site. This may be effected enzymatically by the Mg^{++}-activated IPP-ase. Now, locally resynthesized ATP may recombine with the protein in complex with Mg^{++} released from combination with pyrophosphate. This type of contraction-relaxation cycle, resembling a spatially compact reciprocating mechanism, obviates the necessity for invoking "long-range" migrations of reactants. The highly speculative nature of this scheme may eventually be resolved when the mechanism and site of resynthesis of the inorganic pyrophosphate are discovered, although this should not be an insurmountable objection to the working hypothesis, since both pyrophosphate and pyrophosphatase apparently occur in a wide variety of biological systems (*cf.* Bloch-Frankenthal, 1954).

SUMMARY

1. Mytilus sperm tail ATP-ase temperature coefficient $(Q_{10}) = 2$; temperature optimum occurs in the range between 20° and 30° C.

2. The sperm tails actively dephosphorylate sodium pyrophosphate (NaPP) as well as ATP. Two separate enzymes are involved, which together with their substrates apparently compete for the magnesium ions in the medium.

3. $K_{M-ATP-ase} = 3.1 \times 10^{-4}$; $K_{M-IPP-ase} = 4.3 \times 10^{-5}$.

4. In low concentrations of ATP and NaPP, the amount of inorganic phosphate liberated is additive, while at higher concentrations, inhibition occurs.

5. ATP-ase is more sensitive than IPP-ase to sulfhydryl inhibition, although iodoacetate has only slight effect on both enzymes.

6. An hypothesis is proposed that regulation of the undulatory flagellar wave primarily resides within the flagellum itself, and is a function of the reciprocal

activity of two enzymes, ATP-ase in the contraction phase, and IPP-ase in the relaxation phase.

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VASCULAR BUDDING IN BOTRYLLOIDES[1, 2]

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In our previous paper (1957) it has been demonstrated that in *Botryllus primigenus*, in addition to the ordinary palleal (peribranchial) budding, new buds are formed also from aggregations of blood-cells at the base of ampullae, and the epithet "vascular" has been proposed for that kind of budding. The question at once arises whether this vascular budding occurs also in other members of the family Botryllidae. Our researches along this line have revealed that *Botrylloides violaceum* under certain circumstances propagates by vascular budding entirely analogous to that of *Botryllus*.

In this brief note the process of vascular budding in *Botrylloides* will be described and then compared with that of *Botryllus*.

MATERIALS AND METHODS

The materials on which the following observations were made were living colonies of *Botrylloides violaceum* Oka, commonly found in the vicinity of Shimoda Marine Biological Station, Shimoda, Japan. As is well known, in *Botrylloides* the zooids are arranged in meandering systems instead of in circular systems as in *Botryllus*.

To facilitate observation, colonies were fixed on glass slides. The technique used for fixing the colonies, setting out the slides in the bay, etc. was essentially the same as that described in the paper of Oka and Usui (1944). Observations on living materials were supplemented, if necessary, with examination of sections.

We take this opportunity and express our thanks to the Director and staff of the Station for providing us facilities for carrying out this research. Thanks are also due to Miss Yoshiko Oshima for her helpful assistance in laboratory works.

OBSERVATIONS

Developmental cycle in the colony of Botrylloides

Developmental cycle in the colony of *Botrylloides* is exactly the same as in *Botryllus*. In both, the zooids in a colony are perfectly coordinated, so we can speak of the phases of a colony as a whole. A colony has four successive phases, which constitute a developmental cycle. For particulars see our previous paper (1957).

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[1] Contributions from the Shimoda Marine Biological Station, No. 109.

[2] The cost of this research has been partly covered by the Scientific Research Expenditure of the Department of Education of Japan.

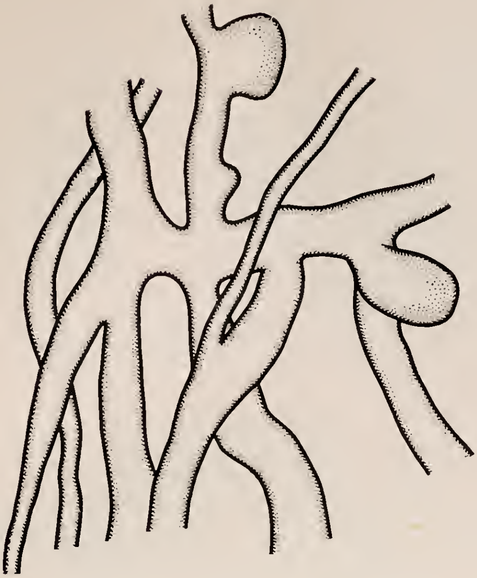

FIGURE 1. Test vessels immediately after isolation. × ca. 60

Conditions for the appearance of vascular buds

In the normal growth of the colony of *Botrylloides violaceum,* buds are formed exclusively from the palleal wall, *i.e.,* no sign of vascular budding is seen. When, however, a small piece containing ampullae and vessels but no zooids is cut out from the marginal part of a colony, vascular buds appear in it after 2 or 3 days.

In *Botrylloides* colonies, as in *Botryllus* colonies, numerous blood vessels traverse the test (Fig. 1) and terminate in contractile ampullae at the periphery of the

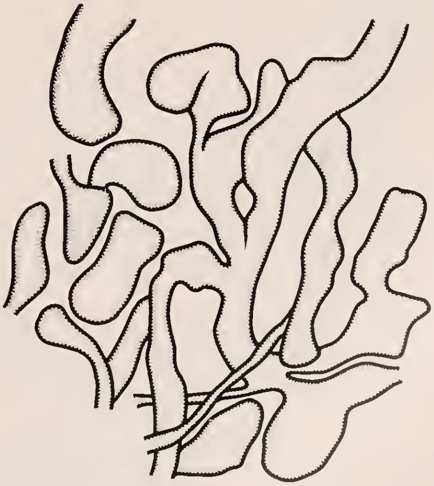

FIGURE 2. Test vessels in a one-day-old piece. × ca. 60

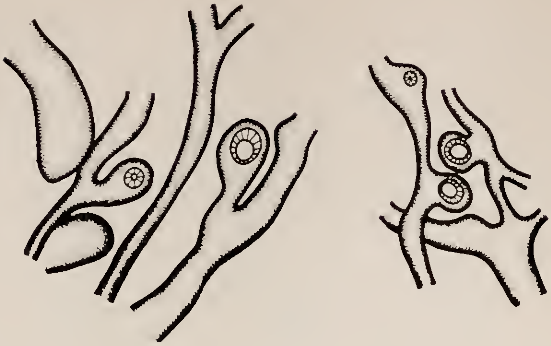

FIGURE 3. Vascular buds in a 3-day-old piece. × ca. 60

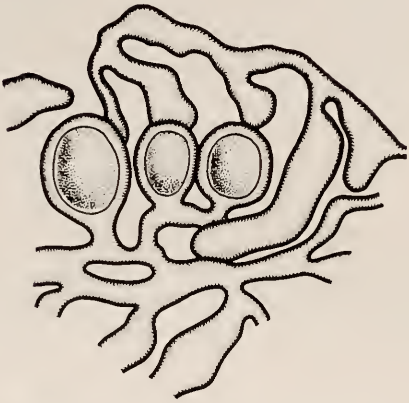

FIGURE 4. Vascular buds in a 4-day-old piece. × ca. 60

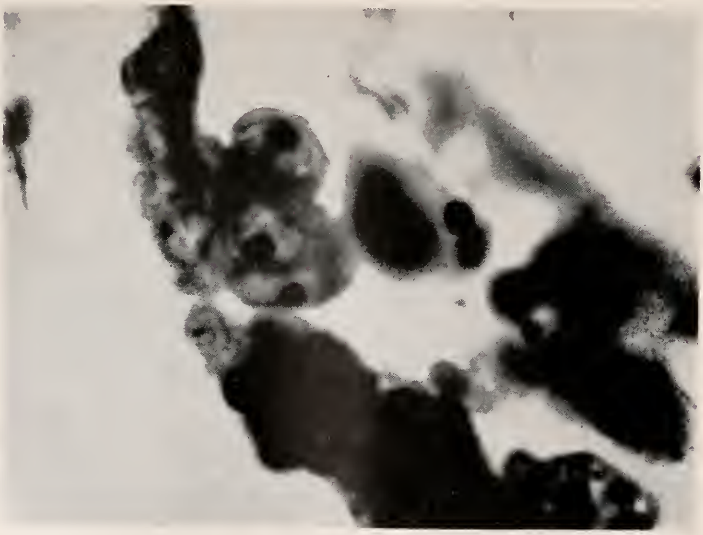

FIGURE 5. Section of a bud from a 2-day-old piece. × ca. 2000

colony. In a cut-out piece, the whole vascular system, inclusive of ampullae, strongly contracts. At the same time many club-shaped branches are sent out, and finally a dense network of anastomosing vessels is formed (Fig. 2). It is seen that a flow of blood is maintained in it. On such vessels the vascular buds are formed.

Formation of the vascular bud and its further development

It is seen in sections that the formation of the vascular bud is initiated by gathering of lymphocytes (diameter ca. 3–4 μ) under the epidermis of the blood vessel (Fig. 5). The initial number of lymphocytes is about 15–20 as in *Botryllus*. The development of a new zooid out of this cell mass follows exactly the same course as in *Botryllus*. At first, through intensive cell division a hollow blastula-like structure (diameter ca. 30–40 μ) is formed (Fig. 6); at the same time, the local epidermis of the blood vessel gradually protrudes so as to wrap up the vesicle in itself (Fig. 3). The bud is now distinctly visible as such even in living materials. Morphologically, the vascular bud of this stage (diameter 50 μ) corresponds to the palleal bud of stage 3 except that it has no ova. Further development is the same as in the palleal bud. As an example, buds in a 4-day-old piece are shown in Figure 4.

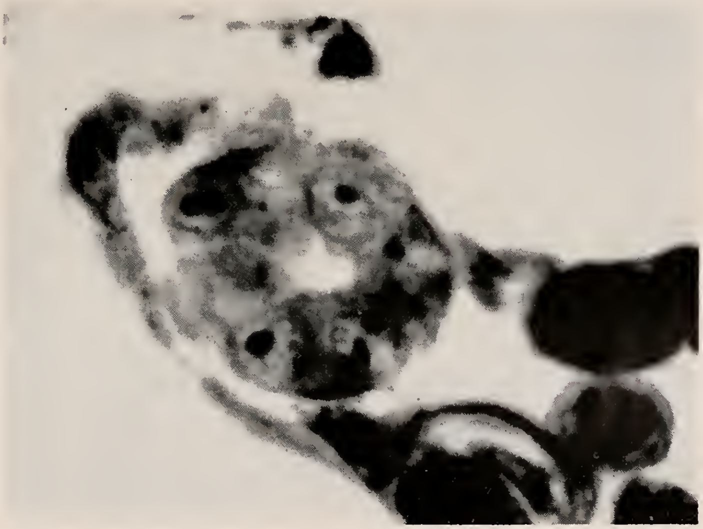

FIGURE 6. Section of a bud from a 3-day-old piece. × ca. 2000

Time of appearance

At whatever phase in the developmental cycle of the colony isolation may occur, vascular budding always begins two to three days after isolation and lasts about 24 hours. This means that vascular budding is not related to any definite phase of the original colony.

Vascular budding is strictly restricted to that period, for, as it seems, further bud-formation from the vascular wall is inhibited by growing vascular buds, and the new zooids, once formed by vascular budding, propagate exclusively by palleal budding.

Site of appearance

At the time of budding, the vascular system in the isolated piece is represented by a network of blood vessels, the diameter of which varies from 45 μ to 120 μ. Unlike *Botryllus,* the vascular buds never appear at the bases of ampullae. Nor do they appear on the newly formed club-shaped endings. They are always formed along the walls of the old blood vessels.

Degeneration of the buds

At the end of the budding period, *i.e.*, 3 to 4 days after isolation, we could often count 60-70 newly formed buds, but not all of them continued to develop. As in the case of *Botryllus,* only those which surpass a certain size can develop and form perfect zooids, while the remaining ones undergo involution. An example of various sizes of buds is shown in Table I.

TABLE I

Various sizes of vascular buds in a piece 4 days after isolation

Diameter (in μ)		Number of buds
ca. 10		9
ca. 15		8
ca. 20		25
ca. 30		17
ca. 40		6
ca. 50		1
ca. 60		1
ca. 70		1
ca. 80		1
ca. 90		1
	Total	70

Of these 70 buds, those larger than 40 μ continued to develop (11 buds, or 16%), while those under 30 μ soon began involution and finally disappeared without leaving any traces behind (59 buds, or 84%). In another case, of 35 buds once formed, only 6 (17%) developed into perfect zooids and formed a colony with two systems. Thereupon the colony began to grow by palleal budding.

DISCUSSION

Differences between Botryllus and Botrylloides

The formation of the bud itself is precisely alike in both forms. Yet, as to the conditions for and the time and site of the appearance of the vascular buds, there are differences between *Botryllus primigenus* and *Botrylloides violaceum* as stated below:

1. In *Botryllus,* vascular budding occurs in active colonies concomitantly with palleal budding. In *Botrylloides,* vascular budding is never seen under normal

conditions. Only when a small piece of a colony devoid of zooids is isolated, vascular budding is, so to speak, evoked in it.

2. In *Botryllus* the appearance of the vascular buds is limited to a certain phase (late phase B) in the developmental cycle of the colony. In *Botrylloides* vascular buds can be formed at any phase of the original colony.

3. In *Botryllus* the buds are located strictly at the bases of the ampullae, while in *Botrylloides* the buds are scattered across the colony along the walls of the vascular system.

Actually all these differences are the same as existing between *Botryllus primigenus* and *Botrylloides gascoi* except on one point. In *Botrylloides violaceum* we could repeatedly observe vascular budding in isolated pieces. In *Botrylloides gascoi,* however, an isolated piece devoid of zooids never regenerated a colony, and none of the ampullae in such a piece showed the least tendency towards budding (Bancroft, 1903, p. 451). Probably this led Bancroft to suppose that the presumable vascular buds observed by him in an aestivating colony were developed not from vessels but from the zooids of the original colony before these had degenerated entirely and later severed their connections with the parent zooids. It is to be hoped that some future investigator will repeat the experiments with *Botrylloides gascoi.*

Regulation acting upon the vascular buds

Botryllids are known for their zooids being most perfectly coordinated.

In *Botryllus,* the vascular buds are formed a little later than the corresponding palleal buds, but they are soon synchronized with these. Buds formed too late are forced to degenerate, thus being eliminated from the colony. All this regulating influence is supposed to come from the pre-existing active zooids.

In *Botrylloides,* the vascular buds are formed in the absence of any pre-existing zooids. Yet, sooner or later, all the newly formed zooids are synchronized, and, as in *Botryllus,* buds formed too late are eliminated from the colony. Possibly with the growth of early buds a new coordinating system is established in the piece and this regulates the growth of late-coming buds.

Budding in Botryllidae

Now that vascular budding has been demonstrated also in *Botrylloides,* it is to be assumed that this kind of budding is rather widely distributed among the family Botryllidae.

It is generally believed that stolonial budding—of which vascular budding is only a special form—is a rather primitive type of budding, while palleal budding is phylogenetically a relatively late acquisition. Moreover, palleal budding is so unique in nature that it cannot be derived from any other known kind of budding. So it has been assumed "that the primitive pleurogonid, undoubtedly derived from an enterogonid, had already lost any such capacity for budding, and that within the new order the Botryllinae have re-acquired it by a new method" (Berrill, 1950, pp. 50–51). That the original capacity for budding has not been completely lost in botryllids is clear from our investigations on *Botryllus* and *Botrylloides.* Only, with the rise of the new method of palleal budding, it has been more and more suppressed. In *Botryllus primigenus* vascular budding still takes part, though concomitantly with palleal budding, in the natural growth of the colony. In

Botrylloides violaceum, and probably *Botrylloides gascoi,* vascular budding is totally suppressed in the ordinary life of the colony. Only in the absence of zooids the otherwise latent capacity of forming buds from the walls of the blood vessels is called forth as a means to save the colony from extinction.

SUMMARY

1. Generally *Botrylloides violaceum* Oka propagates by palleal budding alone. Only when a small piece of a colony devoid of zooids is isolated, new buds are formed from the walls of the test vessels, *i.e.*, vascular budding appears.

2. As in *Botryllus,* these new buds are formed from aggregations of lymphocytes under the wall of the test vessels.

3. Unlike *Botryllus,* the buds are not bound to any definite sites, but are distributed irregularly along the walls of the vascular system.

4. The buds generally appear 2–3 days after isolation, at whatever phase of the original colony the isolation may occur.

5. Major difference between *Botryllus* and *Botrylloides* is that in the former vascular budding coexists with palleal budding, while in the latter vascular budding is totally suppressed in the normal life of the colony.

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AXENIC CULTIVATION OF THE BRINE SHRIMP ARTEMIA SALINA

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In a previous paper (Provasoli, Shiraishi and Lance, 1959) we have added to Gibor's (1956) observations that related species of algal flagellates may be either good or bad food for *Artemia*. This idiosyncrasy may depend upon nutritional deficiencies in the algal food, on toxic metabolic products, or even upon some nutrient in excess. One way to attack this ecological problem is to grow *Artemia* on a non-living medium as a step toward a chemically defined medium and, finally, identification of all its nutritional requirements.

The present paper concerns the first stage, *i.e.*, the growth of *Artemia* on a non-living complex medium.

Materials and Methods

The amphigonic American race of *Artemia salina* was employed. Utah brine shrimp eggs (Aquarium Stock Co. Inc., 31 Warren Street, New York 7, New York) proved more satisfactory than other samples tried in respect to percentage of hatching and speed of development.

Sterilization of the eggs. Durable eggs of *Artemia* obtained commercially always contain many dead dried eggs whose chorion is cracked. These eggs are lighter and cannot be disinfected as rapidly as the intact viable ones and should be eliminated at the onset to avoid infections from the inoculum. The technique of disinfection is a modification of the one employed by Gibor (1956).

The dead eggs, being lighter than the viable ones, are eliminated by the flotations in sea water. The eggs are disinfected in screw cap tubes for 10 minutes in Merthiolate solution (1 : 1000 in H_2O) + 0.2 ml.% of a 10% solution of Aerosol OT. to improve wettability. The disinfectant is decanted and the eggs washed in three baths of sterile sea water. The egg slurry is distributed into several tubes of a sterility-test medium (*STP*, Table I) and allowed to hatch 2–3 days at 22–26° C.). Contamination generally shows in 2–4 days. The new-born nauplii develop to second metanauplii at the expense of the reserves of yolk within 4–7 days; the third metanauplii, if not fed, die.

The metanauplii are transferred into a nutrient medium 1–2 days after the first nauplii have hatched, to secure a more uniform inoculum in respect to age; hatching is spread over several days.

The growing larvae consume the particulate food rapidly and must be transferred approximately every 6–8 days, especially after the fourth stage. Transfers are made with Pasteur pipettes connected to a mouthpiece by rubber tubing, to allow a clear view while fishing the larval forms. The later larval stages and the young adults defy suction unless sucked head first, while swimming toward the tip of the pipette. To avoid air-borne infection we used a transfer hood (top and back glass,

TABLE I

STP medium

Sea water	80 ml.
H_2O	15 ml.
Soil extract	5 ml.
$NaNO_3$	20 mg.
K_2HPO_4	1 mg.
Na H glutamate	50 mg.
Glycine	10 mg.
DL-Alanine	10 mg.
Vitamin No. 8A*	0.1 ml.
Trypticase (B.B.L.)	20 mg.
Yeast autolysate (Albimi)	20 mg.
Sucrose	0.1 g.
pH 7.5	

* See Table 4, p. 408 in Provasoli *et al.* (1957).

open in front) with a "Letheray" germicidal UV lamp (see description and figure in Provasoli, Shiraishi and Lance, 1959).

Preparation of media. The following medium (Table II) allows growth to adulthood.

TABLE II

Complete medium

STP (1)	100 ml.
Cholesterol (2)	200 μg.
Dehydrated liver infusion No. L 25 (3)	100 mg.
Trypticase (B.B.L.)	300 mg.
Alkaline-hydrolysed nucleic acid (4)	40 mg.
Acid-hydrolysed DNA (5)	10 mg.
Sucrose	200 mg.
Vitamins mix Art. II (6)	1 ml.
Paramecium factor (7)	5 mg.
Glutathione (8)	30 mg.
Ascorbic acid (8)	3 mg.
Horse serum (aseptic)	5 ml.
Rice starch (9)	500 mg.
pH 7.5	

(1) See Table I.
(2) Dissolved in ethanol.
(3) Oxoid Ltd., England.
(4) Yeast RNA brought to pH 9.0 with NaOH and steamed for one hour.
(5) Herring DNA brought to pH 1.5–2.5 with H_2SO_4 and steamed for two hours.
(6) Vitamins mix Art. II

Thiamine HCl	10 mg. %
Biotin	0.5 mg. %
Folic acid	7 mg. %
Nicotinic acid	50 mg. %
Choline	500 mg. %
Ca pantothenate	70 mg. %
Pyridoxine HCl	8 mg. %
Carnitine	20 mg. %
Riboflavin	0.1 mg. %

(7) Paramecium factor was kindly supplied by Dr. D. M. Lilly (1 part dried yeast cells + 1 part H_2O, autolyzed at 58–60° C. for two hours; the particles are centrifuged and the supernatant vacuum-dried; to obtain a fairly good suspension, bring to a boil.

(8) Fresh solutions of glutathione and ascorbic acid, sterilized by glass-filtration, are added before inoculation.

At first the starch was added to autoclaved media as a slurry. Rice-starch powder is mixed with glass beads (200 μ diameter-Superbrite type 100, Minnesota Mining and Manuf. Co.) and sterilized for two hours at 180° C.; sterile water is added to form a slurry.

We found later that *Artemia* ingests cooked starch equally well; this eliminated one aseptic addition. To prevent the starch from forming a semi-solid mass during sterilization, 500 mg.% of starch powder is added to the complete medium (minus other aseptic additions) before sterilization; the medium is brought to a boil while being stirred vigorously with a glass rod or on a heating plate equipped with a magnetic stirrer. The starch, on boiling, forms small floccules which become larger upon cooling but remain acceptable to *Artemia*. The medium is then tubed and autoclaved.

Miscellaneous preparations:

a) The cholesterol is generally added as an alcohol solution. It has also been employed absorbed on cellulose powder ("Celluflour," Turtox) following the techniques of Singh and Brown (1957) (1 mg. of cholesterol dissolved in 10 ml. ether is mixed with 200 mg. Celluflour; let dry, then added to media before autoclaving).

b) Fatty acids absorbed on starch: 980 mg. of the following fatty acid mixture (proportions of House and Barlow, 1956) palmitic acid 200 mg. + stearic acid 100 mg. + oleic acid 480 mg. + linoleic acid 150 mg. + linolenic 50 mg. are dissolved in ether and mixed with 10 g. of starch; after evaporation of the ether, the powder is sterilized in ethylene oxide for twelve hours; the powder is made aseptically into a slurry with water, and a solution of NaOH or $Ca(OH)_2$ added to neutralize the fatty acids.

c) Albumen (bovine) fraction V (N.B.C.) is dissolved in sea water, glass-filter-sterilized, and added aseptically.

Fraction V was employed also as a carrier of cholesterol and the fatty acids mixture. Sterile solutions of fraction V and cholesterol (dissolved in ethanol) are mixed and added after autoclaving. Two ml. of a sterile 8% solution of Fraction V were mixed aseptically with 2 ml. of an autoclaved fatty acid mixture (dissolve in 50 ml. of H_2O, 1 ml. of concentrated NaOH, stearic acid 24 mg., palmitic acid 54 mg., linoleic acid 36 mg., linolenic acid 12 mg., oleic acid 0.12 ml., adjust to pH 8.0 with HCl, autoclave).

INTERPRETATION OF RESULTS

To compare the nutritive value of different foods and supplements we needed to recognize the various larval forms by characters easily visible by inspection of the tubes with a hand lens. We could not resort to the fine external morphological characters employed by Heath (1924) to divide in 13–15 instars the development of *Artemia* from egg to adult; these differences can be evaluated only with a microscope. We decided therefore to employ, slightly modified, the arbitrary nomenclature of Barigozzi (1939) which divides the life-cycle in stages of development clearly distinguishable with the hand lens.

The nauplius is small, roundish, yellow-pink (first instar of Heath). Metanauplius I is small, triangular, yellowish (second instar of Heath). Metanauplius II is similar but bigger (third instar of Heath).

Metanauplius III. "Small" III T-shaped, longer, thin, shows a visible segmentation in the upper thoracic region (fourth instar of Heath); "big" III (1.5 mm.); the first 3–5 thoracic limbs are well developed but do not move well (fifth instar of Heath). The first three metanauplii stages are easily distinguished from the other stages, even with the naked eye, by their jerky swimming: at these stages propulsion depends upon the characteristic backward and forward paddle-like movement of the long second antennae.

Metanauplius IV. "Small" IV (2 mm.). The first thoracic limbs are now moving well and the movement of *Artemia* is a combination of jerks and swimming in circles (sixth instar of Heath); "medium" IV (2.5 mm.) swims on its back gracefully in circles; most of the thoracic limbs are fully articulated and move rhythmically like rippling waves (seventh instar of Heath); "big" IV (3.5 mm.) bigger in size, abdomen longer and slender, the second antennae smaller than the limbs and lying parallel to the head (eighth instar of Heath).

"Juveniles" (5–7 mm.) have stalked eyes, the abdomen elongates and becomes segmented, at the tip of the abdomen the furca becomes evident; they have a slender appearance and resemble adults but are much shorter (ninth-eleventh instars of Heath).

Adults (7–10 mm.). The males have long claspers (modified overgrown second antennae); the females have a slender head and a conspicuous egg-pouch right below the last pair of limbs (twelfth-fifteenth instars of Heath).

We record twice a week the stage reached in each tube. As in many insects, some phases of the life-cycle of *Artemia* seem more critical than others: the transition from the third to the fourth stage and the one between "big IV" to "juveniles." In general, a good way to evaluate the effect of the different supplements is to compare (a) the days required to reach the "small IV" metanauplius, (b) the days needed to reach adulthood, and, if they do not become adults, (c) the stage reached at death and days elapsed since birth.

We generally inoculate 5–8 larvae per tube. Not all develop into adults even in the best media although in these media most do. Quite often, especially from the III metanauplius onward, "black disease" develops: black spots, consisting of fine melanin granules, develop at the lobes of the phyllopodia, especially on the dactylopodite. The incidence of black-spotted individuals is sporadic and could not be correlated with any particular nutritional deficiency; it might be simply a difficulty in molting, *i.e.,* left-over parts of the previous cuticle may impede normal development. We cannot say how much these spots affect normal growth and if they are harmful; in complete media (Table II) the adults often had black spots from the IV metanauplius on, yet they could become adults. Black disease is often common; until its causes and effect on the health of the larvae are known it is impossible to use accurately the percentage survival of a mixed population of normal and black-spotted larvae as an index of the nutritional status.

RESULTS

a) Particulates

Artemia is a voracious particle-feeder as we amply observed when rearing them on living flagellates. We thought that this behavior could be exploited to increase the ratio of ingestion (drinking) of the nutrients added as solutes, because little absorption can be expected by an arthropod except from the middle intestine, the rest of the body being clad in chitin. An ideal situation would be to have a nutritionally inert attractive particle and to supply nutrients as solutes, thus permitting the application of the usual microbiological techniques for replacing complex organic substances with chemically defined components. We tried a variety of particles, many of them nutritionally rich, because we did not know whether the organisms could withstand a medium rich enough in solutes to support their growth. Early experiments had shown that 0.6% Trypticase inhibits *Artemia* and that *Tigriopus* is even more sensitive to organic solutes. The following compounds were ground fine (between 5–20 μ) in a colloidal mill, sterilized by dry heat, and added aseptically as water suspensions to the liquid part of the medium. The liquid (STP, Table I, + a vitamin mix, and 100 mg.% of "Oxoid" liver infusion, no. L 25) employed at the time is nutritionally deficient and, in the absence of particles, *Artemia* grow only up to medium-sized III metanauplii. Additions of 150 mg.% of particulate blood fibrin, yeast cells, corn protein, lactalbumen, Cerophyll, casein, Celluflour, and rice polishings were ineffective, while fish meal or gluten permitted reaching the IV in 23–25 days; rice starch did so in 35–40 days. Rice starch was selected because it is, if digested, mainly a carbon source. It is almost devoid of impurities of other important nutrilites (as is gluten) and therefore offers the possibility of defining requirements for amino acids, proteins, fats, and vitamins.

Larval forms of *Artemia* are voracious: suspended particles are quickly transformed into fecal pellets. We therefore raised the particulate starch to 0.5% and kept it suspended as much as possible by shaking and homogenizing the medium twice daily. Later on, when solutions allowed growth beyond the third metanauplius, we had to transfer the growing metanauplii every 7 days to a fresh medium and to increase the volume of the medium from 5 ml. to 10 ml. Five ml. of medium in 20 × 125 mm. tubes are better for the growth of young larvae (up to the early stages of "small IV") because such larvae swim poorly, feed mostly at the bottom, and need a medium well aerated by a large surface exposed to the air. Later stages are continuously swimming and stirring the medium. Perhaps the later larval stages would grow faster if the media were changed even more often, but this requires much patience and increases contamination (see "Methods"). It was found later that the starch can be added before sterilization if it is precooked while stirring the medium (see "Preparation of Media"); the resulting floccules are still ingested, and remain more easily in suspension.

When we found a medium allowing growth to adulthood (Table II), we reinvestigated the necessity of particles. The liquid part of the medium (excepting the heat-sensitive components of the medium) was autoclaved, filtered through paper, then glass-filter-sterilized, and dispensed aseptically into tubes. Sterile solutions of glutathione, ascorbic acid, and the serum were added last.

This medium is clear and devoid of visible particles. The nauplii of *Artemia* in this medium reached at best the stage of "big III" metanauplii. In the same batch of medium to which was added aseptically a sterile starch slurry, *Artemia* reached adulthood. A similar experiment was done recently but with another medium allowing growth to adulthood; again the absence of particles prevented growth beyond the third metanauplius.

b) Solutes

Trypticase and nucleic acids. In preliminary experiments it was soon discovered that addition of Trypticase (0.3%) and nucleic acids to the liver extract speeded growth greatly; the IV metanauplius stage was reached in 12–19 days but growth stopped at medium-size metanauplii. Whole blood (1 ml./100) and a suspension of red blood cells, as substitutes for the starch particles, did not speed growth or allow a more advanced stage; yeast cells (autoclaved) were inhibitory.

Vitamins. Since the level of the vitamin mixture initially used (Table I) was far below the levels for insects, we suspected that the medium was mainly deficient in vitamins. Tentatively, we chose concentrations and ratios similar to those employed for insects, but at the lower limits because in earlier experiments we found that cholesterol was already inhibitory at values which are low for many insects. Biotin, pyridoxine, folic acid, nicotinic acid, and choline, added singly and in combinations, either affected general vitality (*i.e.*, vigorous swimming), speeded the time required for reaching the fourth metanauplius stage, or permitted growth up to "very big" IV metanauplii. Therefore we designed richer and more complete vitamin mixtures (see latest in Table II). To see whether some vitamins were present in suboptimal or toxic concentrations, we removed singly each vitamin and added it at different concentrations. Thiamine and folic acid proved limiting, indicating that Trypticase, liver extract, and serum at the levels employed in the complete medium (Table II) are inadequate sources of these vitamins for *Artemia*. Adulthood was reached in the complete medium and no inhibitions were found up to the following maximal concentrations tried (wt./100 ml. final medium): thiamine 200 μg., biotin 30 μg., folic acid 300 μg., nicotinic acid 1 mg., pantothenic acid 3 mg. Choline did become inhibitory between 3 and 10 mg., pyridoxine between 50 and 100 μg.%, and riboflavin between 0.1 and 1.0 mg.%.

Serum, glutathione, and paramecium factor. Adults were not obtained until horse serum, glutathione, and paramecium factor were added to the medium. Serum alone reduced the time to reach the IV metanauplius from 29 days (no serum) to 19 days (2 ml./100), and to 13 days (4–10 ml./100); increasing the serum allowed growth up to "big" IV metanauplii.

In the absence of serum, 20–40 mg.% of glutathione enabled the metanauplii to reach the IV stage in 16 days and become "small" IV. Paramecium factor alone or in combination with glutathione seems ineffective, but when added to the combination glutathione plus serum, allows adulthood. Under our conditions, only "very big" IV or juveniles were produced by serum + glutathione; this combination was quite effective in speeding growth; the IV metanauplius was reached in 13 days. For production of adults the serum should reach the level of 3 ml./100 or more (up to 10 ml./100); when the serum was below 3 ml./100, depending on the quantities of serum added, only "big," "medium," or "small" IV metanauplii were produced.

Cysteine can substitute for glutathione in eliciting adulthood: 10 mg.% cysteine was as effective as 20–30 mg.% glutathione.

Horse serum can be substituted with a filter-sterilized solution of dried beef serum (Difco).

The active factors present in serum are heat-resistant: both supernatants of the autoclaved horse or Difco beef serum, glass-filter-sterilized and added aseptically, are as effective as serum.

Cholesterol and fatty acids. Some attempts were made to replace serum. Five milliliters of serum supply, *inter alia*, much neutral fat, lecithin, cephalin, and cholesterol. The medium without serum, except for the concentrations brought in possibly by 100 mg. of Oxoid liver, has no fatty acids and only 200 μg.% cholesterol. Some insects require long-chain unsaturated fatty acids (linoleic acid, linolenic acid); all require cholesterol. Early experiments had shown that cholesterol above 500 μg.% becomes inhibitory, but these experiments were done with poor media. In comparable media, linoleic acid was indifferent at 1 mg.% and inhibitory at 10 mg.%. With better media, cholesterol absorbed on Celluflour inhibited at or above 6 mg.% and linolenic acid was inhibitory above 1 mg.% and became rapidly toxic. Neither cholesterol or linolenic acid replaced the serum. The fatty-acid mixture absorbed onto starch powder was indifferent at 2–5 mg.% and quite toxic above. Thinking that the toxicity might be caused by the acidity of the acids, the sterile fatty-acid starch slurry was adjusted with NaOH or $Ca(OH)_2$; the Na salts are far more toxic than the Ca salts.

Cholesterol, or the fatty acid mixed with serum albumen fraction V, also did not replace serum. However, 200 mg.% of fraction V alone allowed normal adults in two months instead of 25 days; growth up to the IV metanauplius was as in serum. Soya lecithin became rapidly toxic; "Gliddex I" (a refined "lecithin" containing 4% lecithin, 29% cephalin, 55% inositol phosphatides, and 4% soybean oil) absorbed either on starch or casein, is well tolerated up to 10–15 mg.%. However neither lecithin, Gliddex I or other "lecithins" replaced serum.

DISCUSSION

While *Artemia* can be grown without living food and tolerates concentrations of solutes sufficient to permit development to adults, it does not grow wholly on solutes. The particles are needed to stimulate filter-feeding, thus allowing ingestion of enough nutrient solutes for a normal growth rate.

The studies of Croghan (1958a, 1958b) on osmotic regulation in *Artemia* show: *a*) that the cuticle of the branchiae (metepipodites) of the first 10 pairs of the phyllopods is the only part of the external body cuticle that is appreciably permeable in the adults; in the young larvae, where the phyllopods have not yet developed, the neck organ is the permeable region; *b*) the branchiae are the site of active NaCl excretion; *c*) the gut epithelium controls internal water balance by water uptake; *d*) *Artemia* continues to swallow the medium even when devoid of particles. The swallowing behavior of the adults was indicated by the red coloration of the gut walls a few hours after *Artemia* were put into a filtered phenol red solution.

Our experiments show that the rate of swallowing of a particle-free medium is probably very low in the early metanauplii stages—certainly insufficient to provide enough nutrient solutes for growth. Particles stimulate swallowing. Since all the nutrients in our media are in solution, Trager's (1936) conclusion, based on *Aedes aegypti,* that solutes are utilized for growth, applies to Crustacea. Although other invertebrates, like some ciliates, can be grown on solutes, perhaps some phagotrophs, including Crustacea, living in oligotrophic waters, may be obligate or partial phagotrophs because particulate feeding, besides increasing the ingestion of nutrient solutes, does not affect their inability to withstand concentrations of organic solutes high enough to support growth (Provasoli, 1956).

The medium allowing growth to adults (Table II) is still quite complex; it offers, however, more possibilities of dissection than the only other known axenic medium for a crustacean—the blood-glucose mixture of Treillard (1924) for *Daphnia.* It was exciting to have started with an inadequate medium because each experiment permitted the demonstration of some nutritional needs, some of them reflecting obvious requirements, as was the effect of Trypticase and nucleic acids. It is remarkable that thiamine and folic acid were required even in the presence of Trypticase and liver extract which are ordinarily adequate sources of these vitamins. Glutathione is also required and was replaceable by cysteine. So far only another arthropod, the mosquito *Aedes aegyptii,* requires glutathione (Singh and Brown, 1957), even in the presence of adequate cysteine. Interestingly, the "feeding reaction" of *Hydra* is controlled by glutathione which acts as a specific "feeding hormone"—it is not replaceable by cysteine, ascorbic acid or other donors of SH groups (Loomis, 1955).

Serum is required for the full development of adults. The active components of serum are heat-stable. We could not replace serum with mixtures of fats and cholesterol. These results are only indicative: lack of effect may be due to toxicities of some components of the fatty acid mixture or failure to avoid toxicity by presenting them to *Artemia* on the proper fat carrier.

However, if the toxicity of fatty acids is the cause, it might explain some of the nutritional idiosyncrasies found previously (Provasoli, Shiraishi and Lance, 1959). Utilization of flagellates as food may depend as much upon their providing *Artemia* with all the nutrilites needed as with their lacking toxic substances and vice versa when they are not utilized as food.

For *Artemia* we found that especially in the *Chlorophyta* several species, even strains, were inadequate as food while others were not. Chlorophytes are known to produce toxic unsaturated fatty acids such as chlorellin (Spoehr *et al.,* 1949). Indeed this might well be a characteristic of the *Chlorophyta.* Proctor (1957) found that *Haematococcus* is particularly sensitive to palmitic, oleic, and linoleic acids, and also to substances produced by cultures of *Chlamydomonas reinhardii.* The substances produced by *Chlamydomonas* are steam-distillable and fat-like, quite probably a mixture of unsaturated fatty acids. The accumulation of oil droplets is readily observed in different species of *Polytoma,* the colorless counterpart of *Chlamydomonas.* During the logarithmic phase the cells are full of paramylum granules, but as they pass the peak of growth the starch is replaced in great part by fat droplets. This might explain the toxicity of aged cultures of *Chlorella* to *Daphnia magna* found by Ryther (1954).

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SUMMARY

1. *Artemia salina* can be grown aseptically to adults in a non-living medium.

2. The components of the medium are: sea water, Trypticase, liver infusion, hydrolysed RNA and DNA, serum, sucrose, cholesterol, paramecium factor, glutathione, a mixture of B vitamins, and starch particles.

3. Glutathione, thiamine, and folic acid were found essential even in the presence of Trypticase and serum. Glutathione can be replaced by cysteine. Horse or beef serum (Difco) supply unidentified heat-stable nutrients. Cholesterol and mixtures of fatty acids become rapidly toxic, and do not replace serum.

4. *Artemia* is a voracious particle feeder and transforms the starch particles rapidly into fecal pellets. In the absence of starch particles, the liquid part of the medium, though containing all the nutrients, supports growth only to the third-stage metanauplii. This indicates that the rate of ingestion (swallowing) of liquids is too low to support continuous growth and that the particles are necessary to increase the swallowing reaction.

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LARVAL DEVELOPMENT OF THE SAND CRAB EMERITA TALPOIDA (SAY) IN THE LABORATORY [1,2]

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Present knowledge of the larval development of *Emerita talpoida* (Say) is limited to two early reports, both under the generic name *Hippa*. Smith (1877) described three zoeal stages, which he called second, third, and last zoea, and a megalops stage, all from the plankton of Vineyard Sound, Mass. Smith was unable to obtain a first zoea, as females brought into the laboratory invariably cast off their eggs before they hatched. Faxon (1879a) was able to obtain the first zoea from eggs hatched in the laboratory. He was unable to rear any larvae through the first molt into the second stage, but ventured the opinion that one or more stages remained to be discovered between the first and the earliest described by Smith.

The larvae of two other species of *Emerita* have been investigated, *Emerita asiatica* by Menon (1933), and *Emerita analoga* by Johnson and Lewis (1942). Johnson and Lewis were able to obtain the first zoea from eggs hatched in the laboratory, but were unable to maintain the larvae through the first molt. One individual did enter the second stage after 34 days, but died soon afterwards. On the basis of the first zoea obtained in the laboratory and other stages from the plankton, Johnson and Lewis describe five zoeal stages. In addition, they state that a number of specimens were collected which appeared to be intermediate between Stage III and Stage IV, and which they called, for convenience, "Lower Stage IV." Menon (1933) lists five zoeal stages for *Emerita asiatica*, all of which were obtained from the plankton.

The present paper is a description of the larval development of *Emerita talpoida* (Say) based on observations of larvae reared in the laboratory.

The author wishes to express his sincere appreciation to Professor C. G. Bookhout, under whose guidance and direction this work was done and who read and suggested improvements in this manuscript. Thanks are also due to Dr. John D. Costlow, Jr., for his many helpful suggestions during the course of the experiment.

METHODS

Ovigerous females of *Emerita talpoida* were collected on the beach at Fort Macon, N. C., and held in the laboratory in large fingerbowls until hatching occurred. If unmolested, the females remained quiet in the fingerbowls until the time of hatching. At this time they became active and swam in short spurts around the sides of the container. At each spurt of swimming activity a cloud of

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larvae was released. The larvae began actively swimming immediately upon hatching, with no intervening prezoeal stage.

Groups of ten newly hatched larvae were placed in four-inch fingerbowls of sea water which had been filtered through glass wool and inoculated with 200,000 units of penicillin per liter. There were ten such bowls. Other larvae were reared in mass cultures in fingerbowls containing approximately 200 individuals each. Each day the larvae were transferred by means of a pipette to clean bowls of filtered, inoculated sea water. To each bowl each day was added a quantity of *Nitzschia* sp. and newly hatched *Artemia* nauplii. The exception to this was one of the mass culture bowls, to which only *Nitzschia* sp. was added. The zoea were observed to capture and feed upon the *Artemia* nauplii quite readily.

The fingerbowls which originally contained ten larvae each were examined daily and a record made of the number of individuals surviving and the number in each stage of development each day, based upon exuviae found. Larvae in each stage were removed from the mass culture dishes and preserved in 70 per cent alcohol.

Throughout the experiment the larvae were maintained at a temperature of 30.0° C. and under constant illumination from daylight fluorescent lights. The sea water in which the larvae were reared varied in salinity from 28.2 parts per thousand to 35.1 p.p.t., with a mean of 32.2 p.p.t.

RESULTS

In the mass culture dish to which only *Nitzschia* sp. was added all individuals died while still in the first zoeal stage. In the other bowls, to which newly hatched *Artemia* nauplii were added in addition to *Nitzschia*, some of the individuals eventually entered the megalops stage after passing through six or seven zoeal stages. The majority of the individuals which became megalops did so after passing through six distinct zoeal stages; a few individuals went through an additional molt between the sixth zoeal stage and the megalops. The only morphological difference apparent between the sixth zoeal stage and the seventh zoeal stage was an increase of one or two setae on the exopods of the maxillipeds. The appearance of a seventh zoeal stage in a few individuals does indicate, however, that the number of molts through which an individual passes during larval development is not fixed and/or inflexible.

The number of individuals which entered each stage and the average duration of each stage are given in Table I. Although a few deaths occurred during the intermolt period, most of the individuals which died did so at the time of molting. The difficulty in molting was usually the result of the old exuvia adhering to the new exuvia, generally on the maxillipeds and near the tip of the rostral spine. This failure of the old exuvia to detach from the new may be due to a physiological weakness existing in some of the zoea. Whether this weakness also exists in nature is a matter for speculation.

The shortest length of time that it took any individual to become a megalops was 23 days, the longest was 33 days. The average length of the pelagic larval life in the laboratory was 28 days.

With each zoeal molt the number of setae on the exopods of the first and second maxillipeds increased by either one or two. This change in the number of setae

TABLE I

Number of individuals out of the original 100 which entered each stage of development and the average duration of each stage

Stage	I	II	III	IV	V	VI	VII	Megalops
Number of individuals	100	94	75	71	63	45	5	15
Average number of days spent in stage	3	4	3	4	5	9	3	

on the maxillipeds was found to be an accurate indication of the number of molts through which an individual reared in the laboratory had passed.

DESCRIPTION OF THE LARVAE OF E. TALPOIDA

FIRST ZOEA (FIG. 1)

The first zoeal stage is similar to the first stage of *Emerita asiatica*, as described by Menon (1933), and *E. analoga*, as given by Johnson and Lewis (1942). The smoothly rounded carapace is translucent, colorless, and without the lateral spines which are characteristic of subsequent stages. The rostrum is short and broad. The eyes are stalked. The eyestalks are short and thick and lie close against the carapace, directed somewhat posteriorly. The abdomen projects almost straight downward from the carapace and is flexed so that the telson is carried beneath and nearly parallel to the carapace. The exopods of the maxillipeds bear four plumose setae.

Antennules (Fig. 8). These short, unjointed appendages are thick at the base and taper to a blunt point where three setae of about equal length are borne. These setae are slightly longer than the body of the appendage.

Antennae (Fig. 14). The antennae at this stage are rather stubby appendages, produced on the outer side into a spine-like process. From the base of the outer spine there arises a somewhat slenderer dentiform process of about the same length. At the base of this inner process there is a much smaller spine. The form of the antennae is relatively unchanged through the first four zoeal stages, the first indication of a flagellum not appearing until the fifth zoeal stage.

Mandibles (Fig. 20). The mandibles grow out ventrally and then make a

TABLE II

Relative size of larvae in each stage reared in the laboratory. Based on average measurements of 10 or more specimens. Dimensions are given in mm.

Stage	I	II	III	IV	V	VI	Megalops
Max. length of carapace	0.57	0.68	0.88	1.13	1.60	1.90	2.30
Max. width of carapace	0.46	0.56	0.67	0.84	1.00	1.30	1.80
Length of abd. plus telson	0.80	0.84	1.30	1.50	1.80	2.40	1.90
Length of rostrum	0.20	0.68	1.27	1.55	2.40	2.90	—
Length of lateral spine	—	0.30	0.44	0.60	0.80	1.00	—

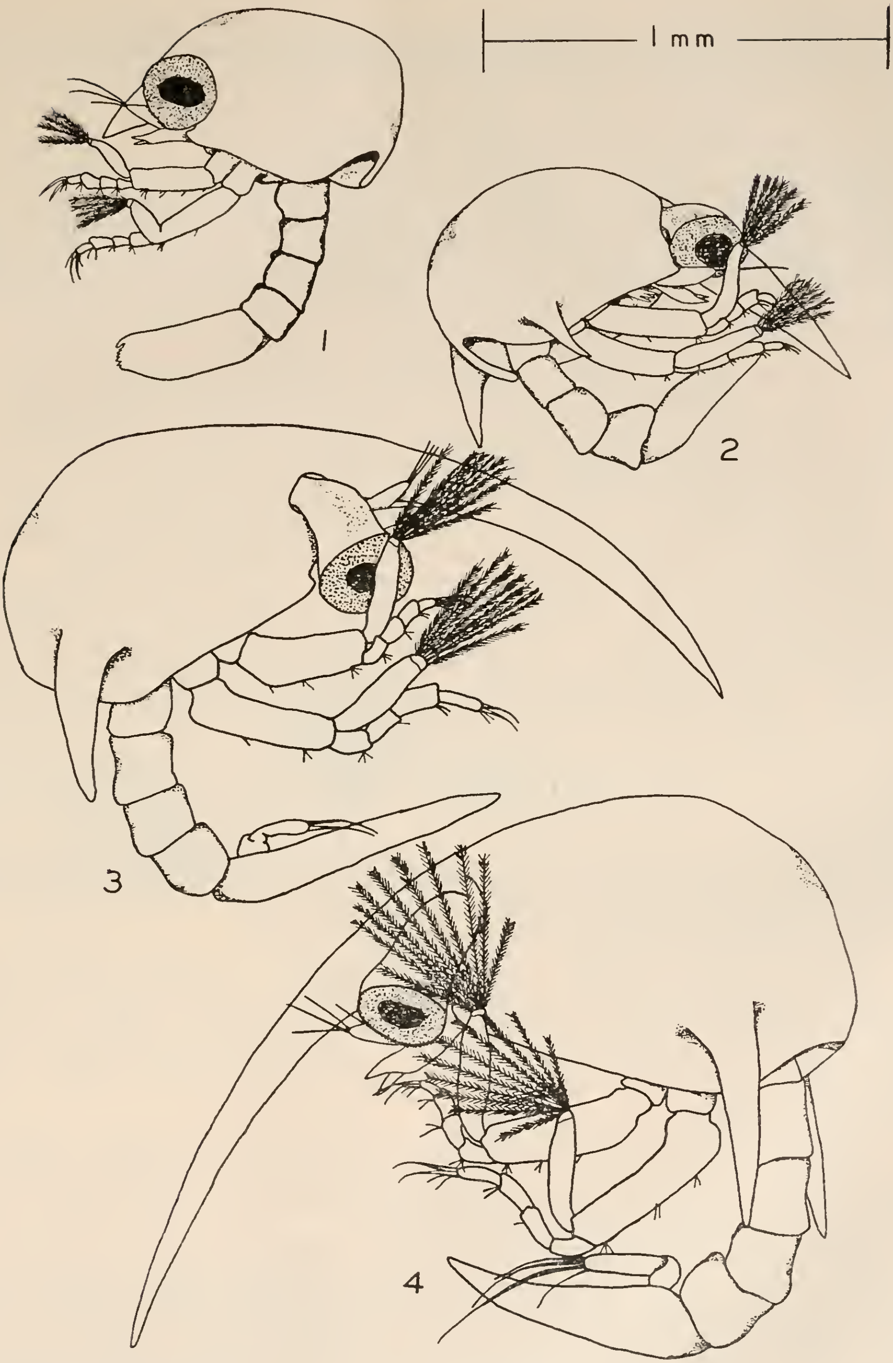

FIGURE 1. First zoea.
FIGURE 2. Second zoea.
FIGURE 3. Third zoea.
FIGURE 4. Fourth zoea.

right angle bend towards the median line so that their crowns are opposed. The crown is armed on its ventral edge by a stout, rather blunt tooth, followed by two shorter, sharp, triangular teeth. Next, there are three or four long slender, setae-like teeth and, finally, a sharp, triangular tooth on the dorsal edge. The entire crown slopes gradually from the ventral to the dorsal edge. These appendages change very little, except for a general increase in size, throughout the zoeal stages.

First maxillae (Fig. 22). These are fleshy appendages, adapted for handling food. The endopod bears three stiff setae at its tip and one short seta about half-way down its inner side. The exopod is twice as large as the endopod and flattened dorso-ventrally. It is divided at its distal end into two tapering horns. Part way down the outer side of the exopod is a small lobe, bearing a single long, curved seta.

Second maxillae (Fig. 24). Each of these appendages is divided into two parts. The protopod is a lobe, tapering anteriorly, where it bears a cluster of three setae. The scaphognathite is sickle-shaped, broader posteriorly than anteriorly, and very thin and foliaceous. Along its anterior-outer margin are nine setae. The posterior and inner margins of the scaphognathite are naked.

First maxillipeds (Fig. 1). These appendages are composed of a short coxopod, a long basipod, a two-segmented exopod and a four-segmented endopod. The basipod bears a group of three setae on its posterior margin just behind the joint with the endopod. One of these setae is shorter and stouter than the other two and armed with minute spines. Behind these is a group of two setae, then a short distance back, a single seta, and finally a single seta close to the joint with the coxopod. The endopod consists of four, cylindrical segments, each bearing setae. The first segment bears three setae just below the joint. One of these is shorter and stouter than the other two and armed with minute spines. The second segment bears two setae just below the joint, one of which is short, stout, and armed with spines as above. The third segment has two setae below the joint. The terminal segment bears four setae at its tip. The outermost two are the longest, curve downward at the ends and bear small spines along their inner margins. The exopod consists of a proximal segment as long as the endopod and a very short terminal segment which bears four long, plumose setae.

Second maxillipeds (Fig. 1). These are very similar to the first maxillipeds except the endopod is somewhat longer than the exopod. The basipod bears three setae along its inner margin; a group of two just behind the joint with the endopod and a single seta about halfway between this and the joint with the coxopod. No rudiments of other thoracic appendages are visible posterior to the second maxillipeds at this stage.

Abdomen. The abdomen is composed of five segments, the first of which is not clearly differentiated from the abdomen at this time. The sixth segment is consolidated with the telson; this becomes apparent when the uropods appear. No rudiments of abdominal appendages are visible.

The telson is slightly broader than long, and faintly concave. The lateral margins curve smoothly to a stout tooth at each side of the posterior margin. The posterior margin of the telson is armed with a complicated series of small spines, with minute denticles between them. The eighth spine from each side is the longest, and between these two longest spines are either nine or ten spines of intermediate length. Thus, in some cases there are twenty-five spines on the posterior margin and in others there are twenty-six. This arrangement holds true for all the zoeal stages, there being sometimes twenty-five and sometimes twenty-six spines on the posterior margin of the telson.

SECOND ZOEA (FIG. 2)

Two lateral spines are now present on the carapace, projecting posteriorly and downward. The rostrum has increased in length tremendously and is now as long as the carapace. The eyestalks are longer and the eyes are carried somewhat farther forward than in the first stage. The exopods of the maxillipeds bear six plumose setae.

Antennules (Fig. 9). Each of these appendages now bears a single stout seta instead of the three which were present in the first stage.

Antennae (Fig. 15). These are the same as in the first zoea.

Mandibles. As in the first zoea.

First maxillae. As in the first zoea except that the exopod bears three long teeth, the outer one showing no articulation at the base.

Second maxillae. As in the first zoea.

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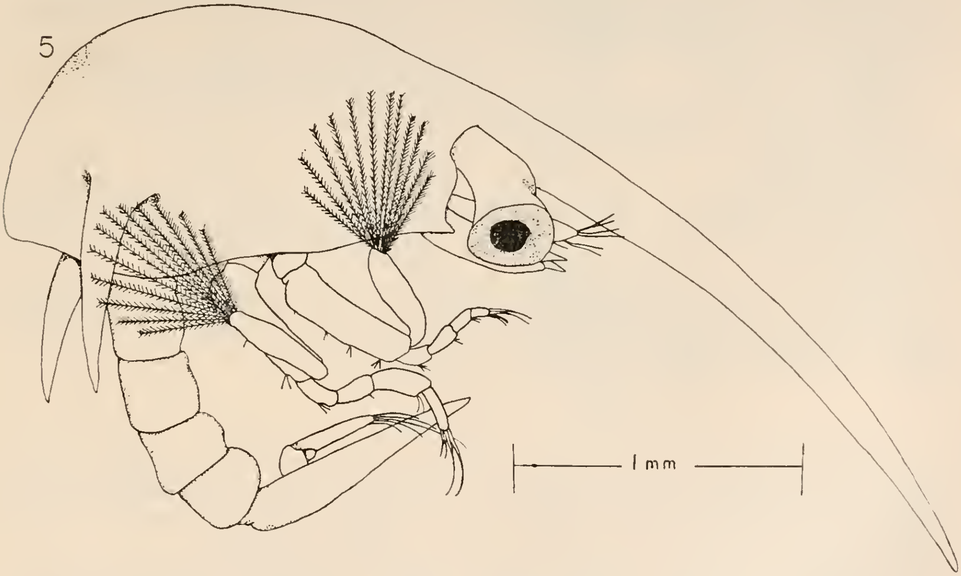


FIGURE 5. Fifth zoea.

THIRD ZOEA (FIG. 3)

The shape of the carapace has changed somewhat. In the first zoea the carapace is practically hemispherical, in the second zoea it is less so, and now in the third zoea, its lateral outline is pear-shaped. The rostrum has continued to lengthen in comparison to the carapace and now exceeds the length of the carapace. Uropods appear on the telson. The exopods of the maxillipeds bear eight plumose setae.

Antennules (Fig. 10). Each of these bears three setae at its tip, much as in the first zoea stage.

Antennae (Fig. 16). As in the first and second zoea.

Mandibles. As in preceding stages.

First maxillae. As in the second zoea.

Second maxillae. As in the first and second zoea, the number of setae on the scaphognathite is nine.

Maxillipeds. The exopods bear eight plumose setae.

Uropods. These appear on the anterior ventral surface of the telson and consist of a short basal segment with a long, flattened lobe extending from it. This lobe is the exopod, as will be seen from later development, and bears two long setae at its tip.

The eyestalks have enlarged and project downward and forward. There is no evidence of any additional thoracic or abdominal appendages at this stage.

FOURTH ZOEA (FIG. 4)

This stage is characterized by the presence of ten plumose setae on the exopods of the maxillipeds and the fact that the uropod bears four setae on its exopod.

Antennules (Fig. 11). Each of these appendages bears four setae, three at the tip and one a short distance down on the inner side.

Antennae (Fig. 17). These are unchanged except for general growth.

Mandibles. These are somewhat slenderer than in preceding stages.

First maxillae. As in preceding stages.

Second maxillae. There are fourteen setae on the anterior-outer margin of the scaphognathite.

Maxillipeds (Fig. 4). These bear ten plumose setae at the tips of the exopods.

Uropods. The exopod bears two long and two short setae. No evidence of endopod as yet.

Abdomen. Each of the four free segments of the abdomen bears two small, round thickenings on its inner side, the evidence of future pleopods. No additional thoracic appendages are visible through the carapace.

FIFTH ZOEA (FIG. 5)

The fifth zoea is characterized by the presence of eleven or twelve plumose setae on the exopods of the maxillipeds, and the appearance of the rudiment of the endopod on the uropods. The rudiments of five future thoracic appendages are now visible through the carapace, posterior to the second maxillipeds.

Antennules (Fig. 12). Each bears six setae; a group of three at the tip, a group of two lower down on the inner margin and a single seta below these.

Antennae (Fig. 18). The rudiment of the flagellum is visible as a conspicuous knob, about half as long as the dentiform process, on the inner side of the antenna.

Mandibles. As in preceding stages.

First maxillae. As in preceding stages.

Second maxillae (Fig. 25). The number of setae along the anterior-outer margin of the scaphognathite has increased to nineteen.

Maxillipeds (Fig. 5). There are eleven or twelve plumose setae on the tips of the exopods. In the first four stages the number of setae on the exopods was constant at four, six, eight, and ten, respectively; now there is some variation. Although twelve appears to be the more usual number, about one-third of the specimens examined had eleven setae on one or more of the maxillipeds. Individuals were found with twelve setae on the first maxilliped of the right side and eleven on the first maxilliped of the left side, and vice versa. This was also found to be true for the second maxillipeds. No individuals, in this stage of development, were found with less than eleven or more than twelve setae on the exopods of the maxillipeds.

Abdomen. As in the fourth zoea.

Uropods. The rudiment of the endopod now appears as a small bud below the exopod. The exopods have increased in length and each now bears five long setae at its tip.

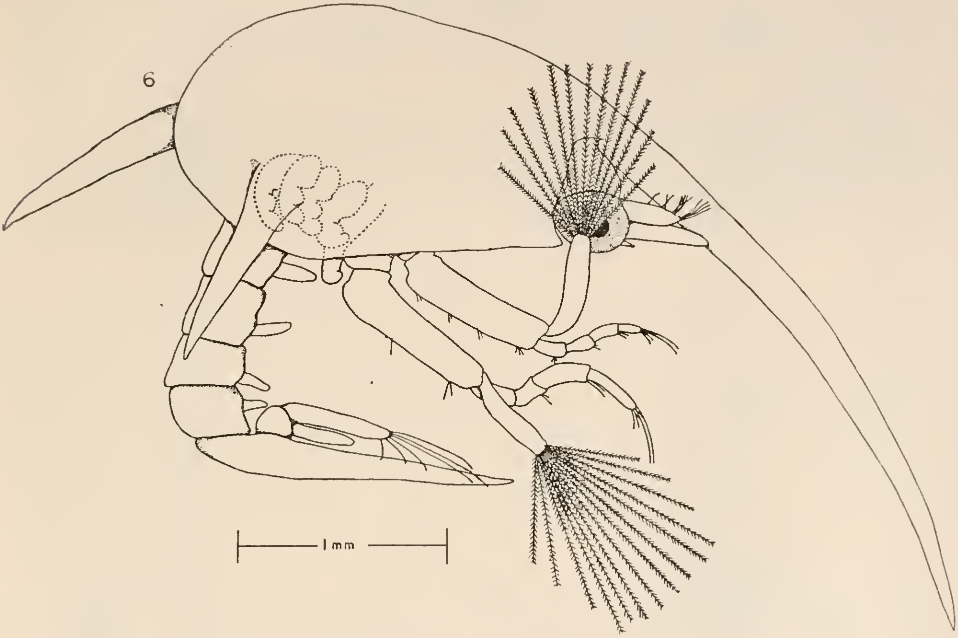


Figure 6. Sixth zoea.

Sixth Zoea (Fig. 6)

There are now uniramous pleopod buds on the four free segments of the abdomen. The exopods of the first and second maxillipeds bear thirteen or fourteen plumose seta. The rostrum continues to increase in length relative to the carapace and is one and one-half times the length of the carapace.

Antennules (Fig. 13). These appendages bear eleven setae in four groups: four at the tip, a group of four below this, a group of two below that, and finally a single seta below these.

Antennae. The flagellum has increased enormously, dwarfing the dentiform processes and extending well beyond the antennule.

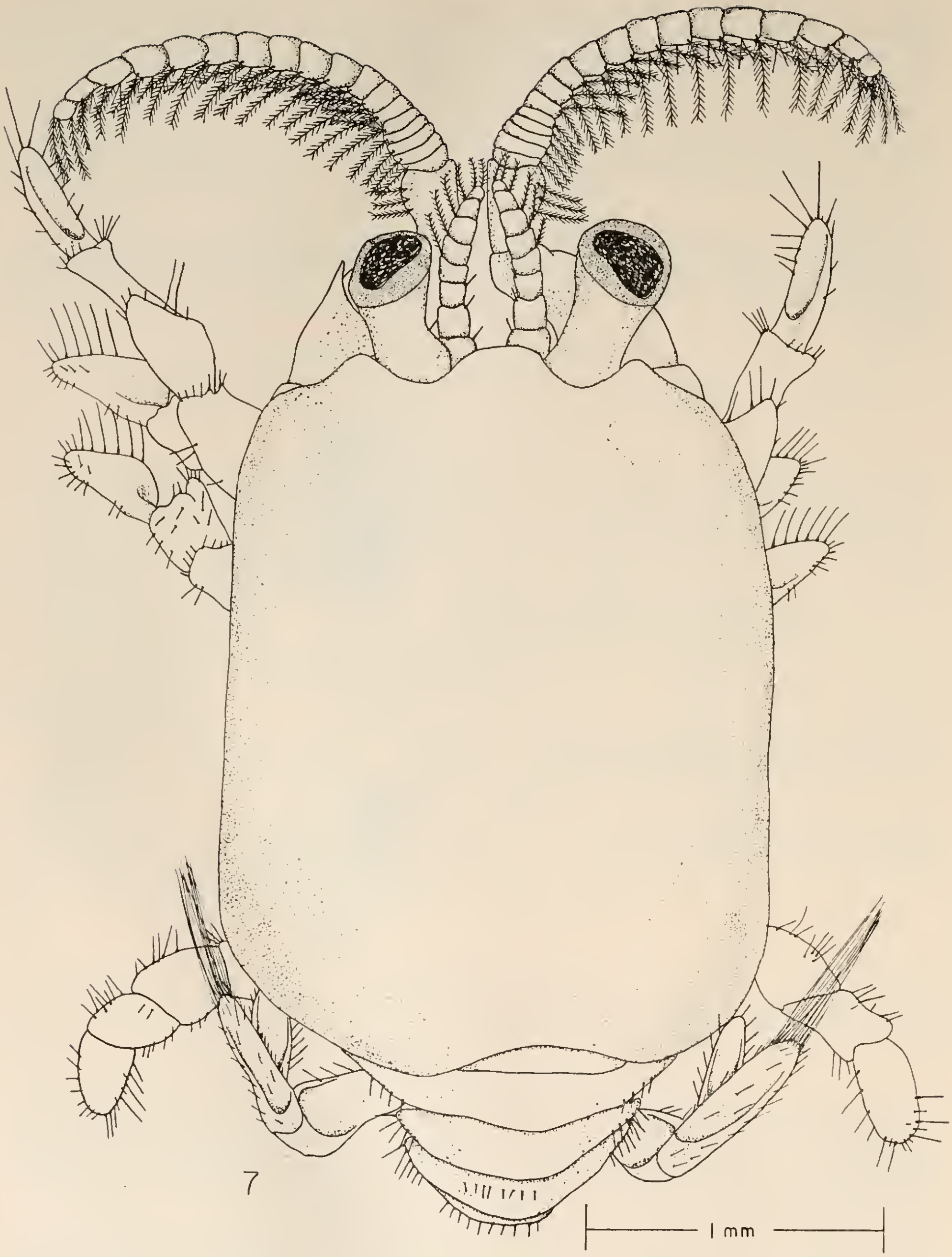


Figure 7. Megalops.

Mandibles (Fig. 21). The crown is armed much as in previous stages, but the shaft of the mandible is not so stout in proportion to its length.

First maxillae (Fig. 23). As in preceding stages.

Second maxillae. There are now 29 setae which extend all the way around the anterior-outer margin of the scaphognathite.

Abdomen. A pair of uniramous, unsegmented pleopods now appears on the second through the fifth segments of the abdomen.

Uropods. The endopod has increased considerably and is now two-thirds the length of the exopod. The exopod bears six setae of unequal length at its tip.

Maxillipeds (Fig. 6). The number of plumose setae on the exopods is now thirteen or fourteen occurring with about equal frequency. No individuals were found with less than thirteen or more than fourteen.

Five thoracic limb buds are plainly visible through the carapace. The first of these is the largest and extends below the edge of the carapace. This is the rudiment of the third maxilliped. The rudiment of the fifth pereiopod is not visible at this time.

Most of the individuals which became megalops did so at the molt following this stage. A few, however, went to a seventh zoea before becoming megalops. The only difference between this seventh zoea and the sixth was the appearance of additional setae on the maxillipeds, making the number fifteen or sixteen.

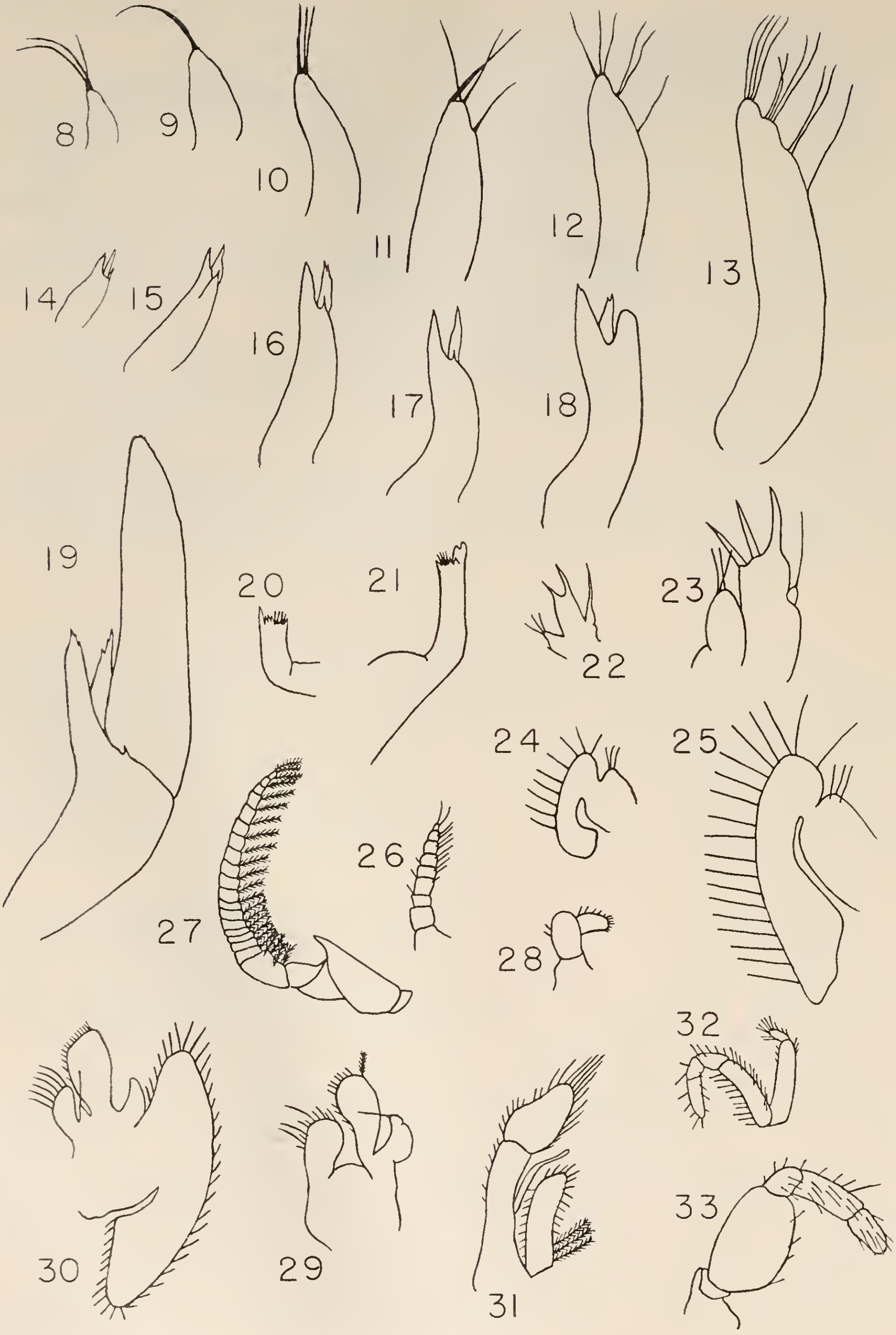
MEGALOPS (FIG. 7)

In general form the megalops resembles the adult, the most obvious difference being that the eyes are still relatively large and the abdomen bears four pairs of pleopods which are quite unlike those of the adult. The megalops carries the abdomen flexed, with the telson between the bases of the pereiopods, though it is not as strongly flexed as in the adult. In swimming the megalops sometimes, but not always, extends the abdomen, thus utilizing the pleopods as swimming appendages. This tendency to swim with the abdomen extended decreased with time and individuals which had been in the megalops stage as long as one day were rarely seen to extend the abdomen.

Antennules (Fig. 26). These appendages are now as long as the peduncles of the antennae and composed of three basal segments and a six-segmented flagellum, which is not noticeably delineated from the basal segments. The first segment bears one, and the succeeding five segments each bear two plumose setae on the outer margin. The segments of the peduncle and the two lower segments of the flagellum may bear a single seta on the inner side. The secondary flagellum of the adult is not present at this stage.

Antennae (Fig. 27). These now possess all the important features of the adult form. There is a scale-like exopod and an endopod composed of three segments and a long flagellum. The flagellum is stout, tapers gradually to a rather blunt tip, and is composed of eighteen segments. The segments are short proximally but gradually increased in length distally until they are longer than broad near the tip. Each segment bears four setae. There are two long, plumose setae which curve inward at their tips, and within these are two shorter, straight, unarmed setae.

Mandibles (Fig. 28). The mandible has undergone a complete change in structure and function. It is no longer an organ of mastication but is adapted, as in



Figures 8–33.

the adult, for the purpose of scraping the antennae and passing food to the mouth. The mandible is now composed of two parts, a broad, foliaceous outer lobe bearing two short, stout setae on its lateral margin, and an inner palp fringed with setae along its anterior and median margins. A very noticeable difference between the appendages of the megalops and those of the adult is the relatively sparse setation of the megalops appendages. In the adult all the appendages are densely fringed with setae.

First maxillae (Fig. 29). The first maxillae now possess all the parts of the adult appendage. The inner lobe (endopod) is broad, bluntly rounded at the tip and armed with stout setae of varying length around the tip and part way down the inner margin. The outer lobe (exopod) is longer and much narrower at the base but broad and round at the tip. It is armed with short, stout setae at the tip and along the inner margin. There is one long plumose seta at the beginning of the outer margin. The palpus is a sac-like lateral projection near the base of the inner lobe. It bears a single long seta at its tip.

Second maxillae (Fig. 30). These appendages are like the adult form except for the relative proportions of the endites and the sparsity of setae as compared to the adult. In the megalops the endites compose three lobes, a small inner lobe bearing long curved setae down its inner margin, a much larger outer lobe fringed with short setae along its anterior-inner margin, and a small papiliform lobe between these two, bearing a single long seta. Between the outer endite and the scaphognathite is a small, triangular lobe representing the endopod. The scaphognathite is broad, thin and has much the same form as in the zoeal stages. It now tapers more acutely anteriorly and the fringe of setae has extended around the broad posterior margin.

First maxilliped (Fig. 31). The anterior segment of the protopod is elongated into a flat, blade-like process bearing setae around its margins, with a series of much longer, plumose setae on the posterior portion of the inner margin. The endopod is represented by a soft, slender lobe arising from near the base of the inner side of the two-segmented exopod. The exopod consists of a long basal segment bearing short setae along its outer margin, and a shorter, broader, paddle-shaped terminal segment bearing very long setae at the tip and shorter setae along its other margins.

Second maxillipeds (Fig. 32). The four-segmented endopod of the second maxilliped differs slightly from the adult form. The third segment makes a right angle bend in the adult, while it is practically straight in the megalops, and the terminal segment is proportionally much shorter and stouter in the megalops than in the adult. The exopod is two-segmented; the basal segment does not taper anteriorly as acutely as in the adult and the oval terminal segment is proportionally smaller in the megalops than in the adult. As in previous cases, the adult appendage is very heavily fringed with setae, while in the megalops the setae are shorter and much sparser.

FIGURES 8–13. Antennule, first to sixth zoea.
FIGURES 14–19. Antenna, first to sixth zoea.
FIGURE 20. Mandible, first zoea.
FIGURE 21. Mandible, sixth zoea.
FIGURE 22. First maxilla, first zoea.
FIGURE 23. First maxilla, sixth zoea.
FIGURE 24. Second maxilla, first zoea.
FIGURE 25. Second maxilla, fifth zoea.
FIGURE 26. Antennule, megalops.
FIGURE 27. Antenna, megalops.
FIGURE 28. Mandible, megalops.
FIGURE 29. First maxilla, megalops.
FIGURE 30. Second maxilla, megalops.
FIGURE 31. First maxilliped, megalops.
FIGURE 32. Second maxilliped, megalops.
FIGURE 33. Third maxilliped, megalops.

Third maxillipeds (Fig. 33). These broad, opercular appendages bear a three-segmented palp at their distal end. The rounded prominence at the articulation of the palpus is lacking in the megalops. The palpus is stouter and the terminal segment much shorter than in the adult.

Pereiopods. The pereiopods are so much like those of the adult that a detailed description of them is unnecessary. The first, second, and third pairs project anteriorly; the fourth pair projects posteriorly and, like the first three pairs, is especially adapted for burrowing in the sand. The fifth pair of pereiopods are very slender, chelate appendages, which are held concealed within the branchial cavity.

Abdomen. The abdomen is, for the first time, composed of six segments, similar in form and proportion to those of the adult, and a triangular telson. The first segment, as seen dorsally, is a small plate, filling a curved sinus in the posterior margin of the carapace. The second segment is the largest of the abdominal segments and is about five times as wide as it is long. Its width is due to a broad lamellar expansion on each side. The third, fourth, and fifth segments are rounded at the outer margins and each is slightly shorter and narrower than the one before. The sixth segment is nearly as wide as the fifth and is as long as it is wide, being the longest of the abdominal segments. The second, third, fourth, and fifth segments of the abdomen each bear a pair of biramous pleopods. The pleopods are made up of three portions; a long basal segment, a paddle-shaped exopod and a knob-like endopod. The pleopods of the second and third segments bear ten plumose setae each on the exopods and those of the fourth and fifth segments bear eleven plumose setae on the exopods. The small endopods increase in length successively from the first to the fourth pleopod, and bear at their tips a series of small hooks. These hooks can engage those of the endopod opposite it, thus joining the pair of pleopods so that they move as one.

Uropods. The appendages of the sixth abdominal segment are essentially the same as those of the adult. There is a two-segmented protopod, the proximal segment of which is short and round and the distal segment, much longer, stouter and flattened. The exopod and endopod are nearly alike: oval, broadly rounded at the tip and fringed with setae which are very long at the tips but shorter along the sides.

Discussion

The first zoea of *Emerita talpoida*, obtained from the egg, corresponds with the first zoea described by Faxon (1879a). The larvae described by Smith (1877) from the plankton as the second, third, and last zoea do not correspond exactly with any of the zoeal stages reared in the laboratory. The results of the present experiments indicate that each zoeal molt results in an increase in the number of setae borne on the exopods of the first and second maxillipeds. In the laboratory their number never increased by more than two setae at any one molt. Using the number of setae on the maxillipeds as an indication of the number of times that an individual has molted, Smith's three zoeae correspond to the third, fourth, and fifth zoeal stages reared in the laboratory. In each case, however, the zoeae from nature possess features (appearance of thoracic limb buds, pleopods, etc.) which show them to be farther advanced in development than the corresponding laboratory stages.

Smith's last zoeal stage bears twelve setae on the exopods of the first and second maxillipeds. A number of individuals in this stage were observed by Smith to change to megalops at a single molt. If setation of the maxillipeds is an accurate index to the number of zoeal molts, then these individuals from nature completed their larval development in five molts, whereas those in the laboratory went through six or seven molts before becoming post-larvae.

Gurney (1942) has questioned the normality of larvae reared in the laboratory and believes that abnormal stages may be reared under artificial conditions. However, Gurney also states that extra stages, through which each individual need not pass in development, occur in nature. Other references to these extra stages have been made by Faxon (1879b), Lebour (1940), Gurney and Lebour (1941), and Broad (1957a). Johnson and Lewis refer to a "Lower Stage IV" in the larvae of *E. analoga,* which was intermediate between Stage III and Stage IV. They could not say whether this was a distinct instar or simply a variable in Stage IV. This is apparently another case of a stage in larval development through which not all individuals pass. If each larval form found in the development of a decapod is described as a stage, then we may expect to find individuals who skip stages or pass through extra stages in the course of normal development, depending upon the number of stages previously defined.

Broad (1957b) working with *Palaemonetes* larvae, has shown that a direct relationship exists between the diet of the larvae and the rate of larval development and frequency of molting. His results show that larvae may respond to sub-optimal conditions of diet by a prolonged larval life and a greater number of larval intermolts. It is probable that a number of other environmental factors also affect the tempo of larval development, and that normal development of *Emerita* and other decapods varies according to the variations in trophic conditions during the breeding season.

SUMMARY

1. *Emerita talpoida* were reared in the laboratory from eggs to the megalops. Of individuals hatched in the laboratory 15 per cent survived to the megalops.

2. The average length of time required to pass through the pelagic larval stages in the laboratory was 28 days. A table is given which shows the average duration of each of the zoeal stages.

3. Six zoeal stages and a megalops are figured and described. A seventh zoeal stage, which was skipped by most of the larvae, is described.

4. Larvae reared in the laboratory are compared with three zoea stages described from the plankton by Smith.

5. The larval development of *Emerita talpoida* should not be regarded as consisting of a fixed number of stages determined by a fixed number of larval intermolts.

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FREE AMINO ACIDS IN SOME AQUATIC INVERTEBRATES[1]

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Free amino acids and many of their derivatives are found present in tissues of all invertebrates so far studied. The distribution of amino acids seems to follow a pattern characteristic for the species. Compounds such as taurine were found in very high concentration in a number of invertebrates. As early as 1904, Kelly reported that *Mytilus edulis* has as much as 1.6 per cent taurine. Other invertebrates contain also large amounts of taurine (Henze, 1905; Mendel, 1904; Kossel and Edlbacher, 1915; Okuda, 1920; Ackermann *et al.*, 1924; Ackermann, 1935; Lewis, 1952; and Kermack *et al.*, 1955). The function of taurine in invertebrates is not known at all, and its mode of formation remains to be elucidated.

The distribution of free amino acids in a number of invertebrates has been studied by Camien *et al.* (1951), Duchâteau and Florkin (1954), Duchâteau *et al.* (1952), and Giordano *et al.* (1950). The amino acids were determined microbiologically. Camien *et al.* found very high concentrations of glycine in muscles of *Homarus vulgaris* and *Maia squinado* and suggested that the role of glycine along with other amino acids was to regulate osmotic pressure. Kermack, Lees and Wood (1955) made an extensive study of the non-protein constituents of the lobster. They found that a large portion of the non-protein nitrogen was accounted for as free alpha-amino nitrogen. The remainder was distributed between trimethylamine oxide, glycine betaine, taurine and volatile bases.

The object of the present study was to determine the pattern of distribution of free amino acids and related substances and to establish a correlation between the pattern of distribution and species and/or environment. The results reported here indicate that such differences do exist in relation to environment and species.

MATERIALS AND METHODS

Animals investigated

The invertebrates investigated include representatives of the following phyla: Coelenterata, Arthropoda, Mollusca, and Echinodermata. The specimens were taken from their environment, rapidly frozen and maintained in this state just prior to analytic procedures (not more than a one-month period). Table I lists these organisms according to phylum and class and shows the location and habitat from which the specimens were taken.

Extraction

Immediately after thawing, the whole organism was quickly weighed and the nitrogenous substances extracted with 80 per cent ethanol according to the pro-

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[1] This work was supported by grants from the Robert A. Welch Foundation, Houston, Texas, and the National Institutes of Health, U. S. Public Health Service.

[2] Part of these data was taken from the M.A. Thesis of John W. Simpson.

TABLE I

Organisms studied and their habitat

Organism*	Location of collection	Habitat
Coelenterata		
Anthozoa		
Bunodosoma cavernata	PA	W
Arthropoda		
Crustacea		
Penaeus aztecus	AB	W
Clibinarius vittatus	SLP	E
Pagurus pollicaris	AB	W
Mollusca		
Gastropoda		
Oliva sayana	SLP	E
Polinices duplicata	AB	W
Thais haemastoma haysae	AB	W
Busycon perversum	AB	W
Fasciolaria distans	PA	W
Siphonaria lineolata	PA	E
Pelecypoda		
Lithophaga bisulcata	PA	W
Crassostrea virginica	SLP	W
Arca umbonata	PA	W
Volsella demissus granosissimus	EBL	E
Cephalopoda		
Loliguncula brevis	AB	W
Echinodermata		
Holothuroidea		
Thyone sp.	SLP	W
Asteroidea		
Luidia clathrata	SLP	W

PA—Port Aransas, Texas
AB—Aransas Bay, near Rockport, Texas
SLP—San Luis Pass, Galveston Island, Texas
EBL—East Beach Lagoon, Galveston Island, Texas
W—Collected directly from marine waters
E—Collected during a period of exposure

* We are indebted to Mr. Howard Lee from the Texas Game and Fish Commission, Rockport, Texas, for permitting the use of equipment necessary for acquisition of organisms.

cedure of Awapara (1948). The amino acids extracted are not produced during the extraction procedure by proteolytic cleavage. Extractions were carried out using live organisms under conditions such as to prevent any enzymatic activity. The live organisms were ground with 80 per cent ethanol as indicated above. Others were frozen and then thawed. Extracts were also prepared as described. Analysis of the extracts revealed that no change had occurred as a result of freezing and thawing.

Fractionation of extractives

Using ion-exchange chromatography the nitrogenous extractives were separated into basic, acidic and neutral substances. Basic substances were separated from neutral and acidic substances on Amberlite CG-50, type 2 H^+, with a screen grading of approximately 200 (passing 200 mesh). The tissue extracts were placed in a beaker containing one gram of the resin and shaken for thirty minutes to allow equilibration with the resin. This suspension was placed in a small column

TABLE II

Amino acids identified

Organism	Al	B-Al	Gly	Ar	As	Glu	Tau	Gla	Pr	OH-Pr	Thr	Tyr	Aspr	His
Coelenterata														
Anthozoa														
Bunodosoma cavernata	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Arthropoda														
Crustacea														
Penaeus aztecus	+	+	+	+	+	+	+	+	+	−	−	+	+	+
Clibinarius vittatus	+	−	+	+	+	+	+	+	+	−	+	+	+	+
Pagurus pollicaris	+	−	+	+	+	+	+	+	+	−	+	+	+	+
Mollusca														
Gastropoda														
Oliva sayana	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Polinices duplicata	+	−	+	+	+	+	+	+	+	+	−	−	−	−
Thais haemastoma haysae	+	−	+	+	+	+	+	+	+	+	−	+	+	+
Busycon perversum	+	−	+	+	+	+	+	+	+	+	−	−	+	+
Fasciolaria distans	+	−	+	+	+	+	+	+	−	−	+	+	+	+
Siphonaria lineolata	+	−	+	+	+	+	+	+	+	+	−	+	+	−
Pelecypoda														
Lithophaga bisulcata	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Crassostrea virginica	+	+	+	+	+	+	+	+	+	−	+	−	−	−
Arca umbonata	+	+	+	+	+	+	+	+	+	−	+	−	−	+
Volsella demissus granosissimus	+	+	+	+	+	+	+	+	+	−	+	+	−	+
Cephalopoda														
Loliguncula brevis	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Echinodermata														
Holothuroidea														
Thyone sp.	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Asteroidea														
Luidia clathrata	+	−	+	+	+	+	+	+	−	−	−	−	−	−

Legend:

+ Present in readily detectable amounts
− Not present in readily detectable amounts

Al—alanine
B-Al—beta alanine
Gly—glycine
Ar—arginine
As—aspartic acid
Glu—glutamic acid
Tau—taurine
Gla—glutamine
Pr—proline
OH-Pr—hydroxyproline
Thr—threonine
Tyr—tyrosine
Aspr—asparagine
His—histidine

(20 × 1 cm.) containing an additional gram of the resin. The neutral and acidic compounds were washed from the resin with 25 cc. water and the basic substances were eluted from the column with 25 cc. 4 N HAc. (Awapara, Davis and Graham, 1959). After removal of the basic substances, taurine and other sulfonic acids were separated from the neutral and acidic amino acids on a column of Dowex-50 H^+. The water wash from the Amberlite was passed slowly through a column containing one gram of Dowex-50 H^+. Taurine was obtained by washing with 25 cc. water and the neutral and acidic amino acids eluted from the resin with 25 cc. 4 N NH_4OH. Each fraction was evaporated to dryness on a steam bath and brought to a 1-cc. volume with water.

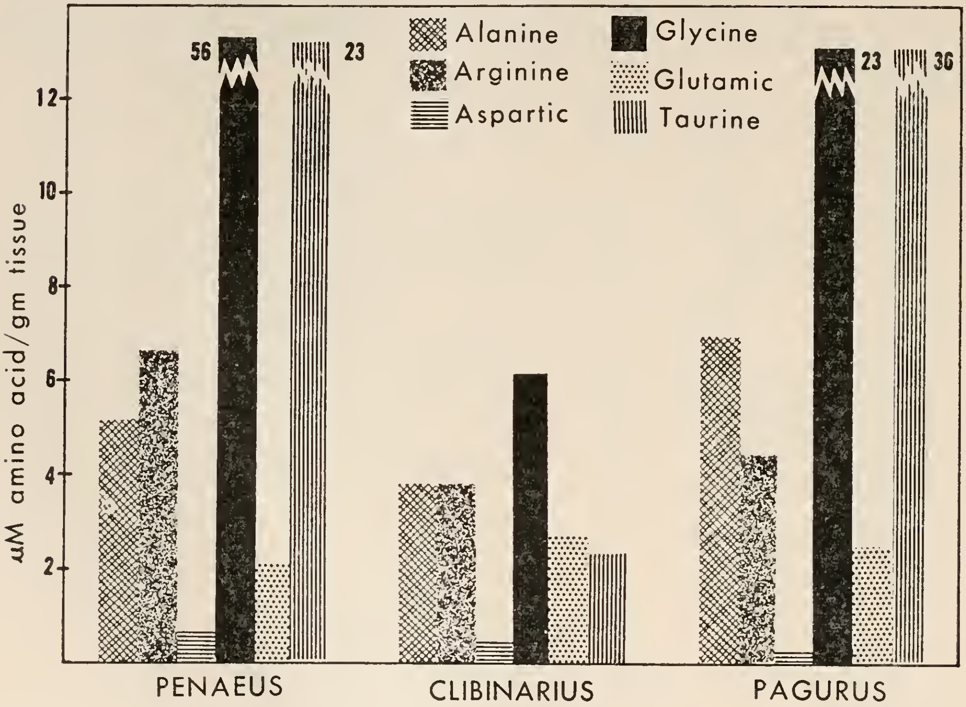

FIGURE 1. Estimated concentration of six amino acids in the tissues of three crustaceans: *Penaeus aztecus*, *Clibinarius vittatus* and *Pagurus pollicaris*.

Paper chromatography

Amino acids of the acidic and neutral fractions were separated by two-dimensional ascending paper partition chromatography. Whatman number 3 MM. filter paper was used and the solvent system employed was phenol-water (72.5 per cent phenol) and 2,4-lutidine-water (62 per cent) in the second direction. The basic amino acids were separated by one-dimensional ascending paper partition chromatography using butanol, acetic acid and water (4 : 1 : 1, by volume) as the solvent.

The amino acid spots were revealed by dipping the paper in a solution of 0.5 per cent ninhydrin in absolute ethanol (w/v).

Identification of amino acids

Amino acids were identified by: 1) their ninhydrin color and position on chromatograms according to previously prepared maps of known substances, and 2) their presence in a particular fraction.

Arginine was further identified by the Sakaguchi reaction, using the alpha napthol reagent described by Acher and Crocker (1952), and histidine by the Pauly's reaction using the sulfanilic acid reagent described by Smith (1958).

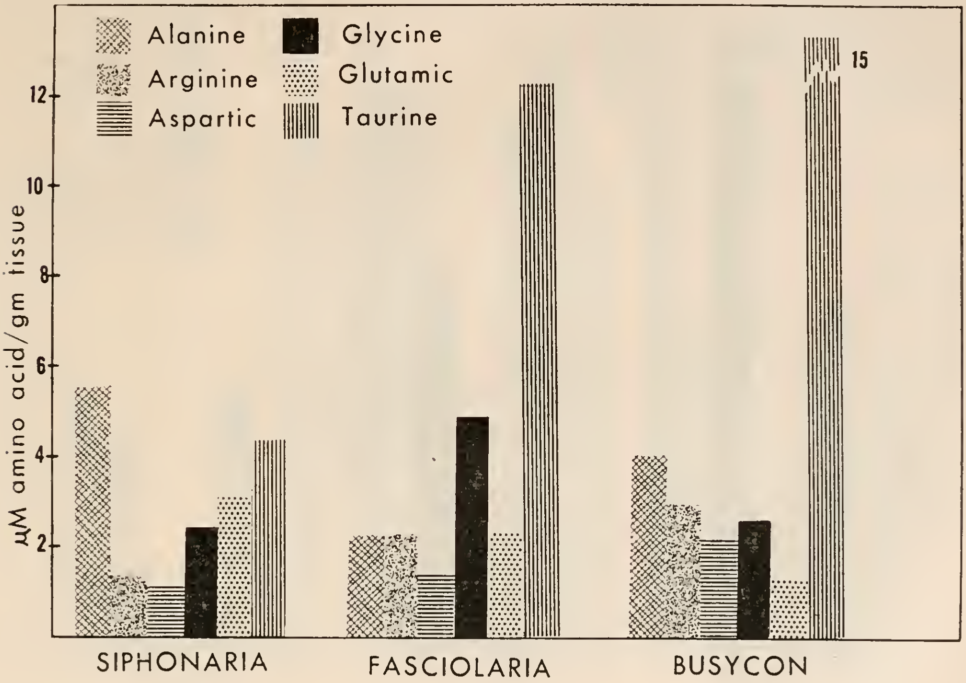

FIGURE 2. Estimated concentration of six amino acids in the tissues of three gastropods: *Siphonaria lineolata*, *Fasciolaria distans* and *Busycon perversum*.

The identification of β-alanine and taurine was supported by preparing chromatograms on paper treated with basic cupric carbonate (Block *et al.*, 1958). These two substances moved to their respective positions on both treated and untreated papers.

Glutamine was further identified by subjecting an aliquot of the extract to hydrolysis with 6 N HCl. Chromatograms of the hydrolysed extracts showed the disappearance of this spot; further, these same chromatograms showed an increase in the glutamic acid spot.

The identification of the asparagine spot was substantiated by elution from undeveloped chromatograms with subsequent acid hydrolysis and re-chromatography of the eluate. Chromatograms of the hydrolysate showed the absence of the spot and the appearance of a spot which corresponded in position and color with the aspartic acid standard.

Estimation of amino acids

Six amino acids which were consistently present were estimated quantitatively. Alanine, aspartic acid, glycine and glutamic acid were measured by the method of Awapara, Landua and Fuerst (1950) with the following modifications suggested by Fowden (1951): 1) the areas of paper containing the spots were cut, placed in test tubes, and 0.2 cc. N NaOH added. The tubes were evacuated for three hours in a desiccator containing concentrated H_2SO_4; 2) additional citrate was included in the ninhydrin reagent of Moore and Stein (1948) equivalent to the amount of NaOH added.

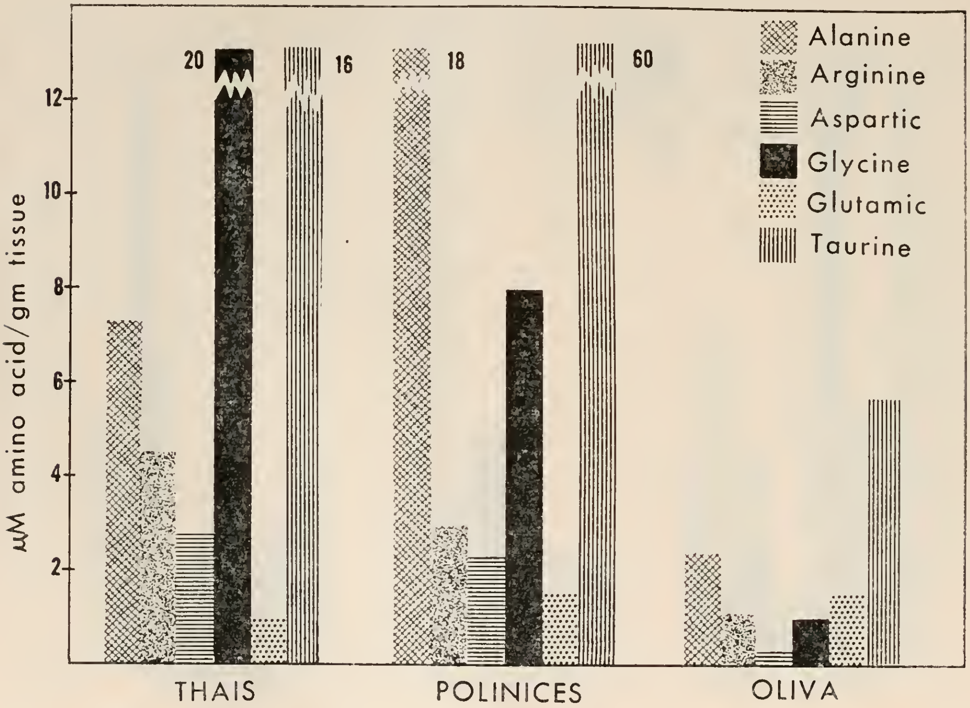

FIGURE 3. Estimated concentration of six amino acids in the tissues of three gastropods: *Thais haemastoma, Polinices duplicata* and *Oliva sayana.*

Taurine was found to be the only ninhydrin-positive material present in the acidic fraction; therefore, this substance was measured directly using the color reagent of Moore and Stein (1948).

Arginine was measured in the extracts by the method of Rosenberg *et al.* (1956). We found no other Sakaguchi-positive material in the extracts as determined by paper chromatography. If other guanidine derivatives were present, their concentration was too low to measure. We can safely assume that nearly all the color in the Rosenberg reaction was due to arginine .

RESULTS AND DISCUSSION

In Table II are shown all the amino acids and related substances detected chromatographically. Other components were present but their identity was not established. In Figures 1 to 6 are shown the concentrations of six amino acids which were found invariably in the species studied. The most striking feature is the high concentration of taurine and glycine in nearly all species studied. The glycine concentration is in many cases equal to that of taurine. The highest concentration of taurine was found in *Penaeus aztecus*, but our value is still considerably lower than the value reported for *Mytilus edulis*. The mode of formation of taurine in invertebrates is not known at all. In mammals it is formed from cysteine after oxidation to cysteine sulfinic acid and decarboxylation of the latter

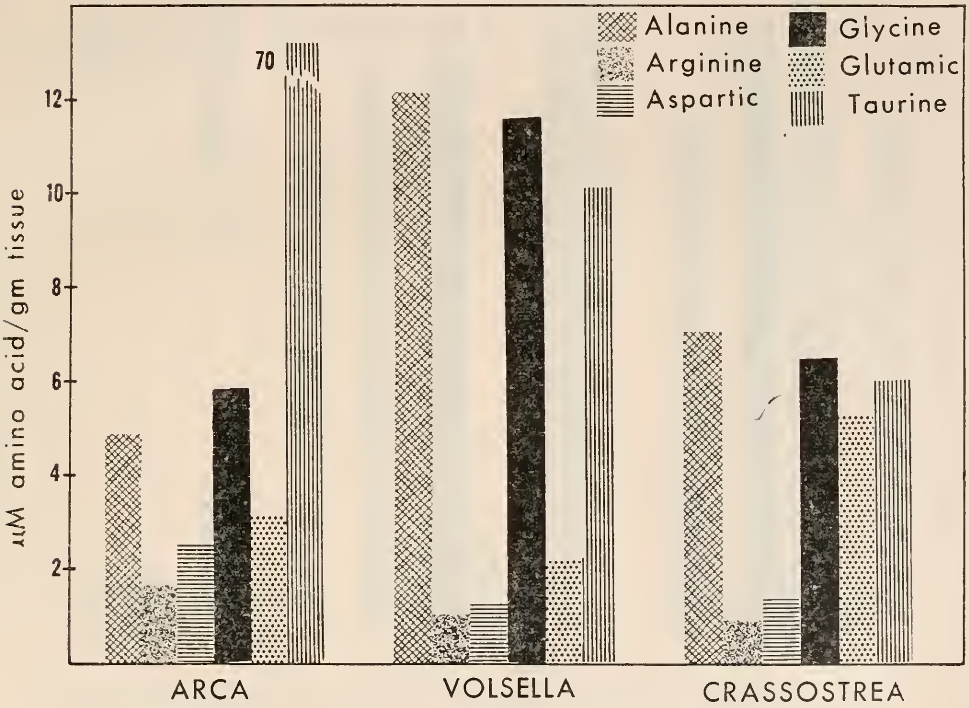

FIGURE 4. Estimated concentration of six amino acids in the tissues of three pelecypods: *Arca umbonata, Volsella demissus granosissimus* and *Crassostrea virginica*.

to hypotaurine (Awapara and Wingo, 1953). Hypotaurine is then oxidized to taurine. Hypotaurine has been reported only once in invertebrates (Shibuya and Ouchi, 1957). The cysteine sulfinic decarboxylase has not been reported in invertebrates. There is the possibility that taurine is not produced by invertebrates but acquired from their diet. Glycine, which is also present in very high amounts, could also be obtained from their diet. One objection to this possibility is the wide range of variation in the taurine concentration of species from the same environment.

Aspartic acid, glutamic acid, and alanine vary much less in concentration than taurine and glycine.These three amino acids are closely linked to the citric acid cycle and their concentration could be easily regulated by the reactions of the cycle. β-alanine was found in some forms but not in others. The only coelenterate which we studied had none. It was found in some Mollusca but not in others. The same was true in the arthropods studied. The function of β-alanine is again unknown. It exists usually as a moiety of carnosine which is known to exist in the muscle of vertebrates and many invertebrates. The problem of formation arises again. β-alanine can be produced by the decarboxylation of aspartic acid, or by the hydrolytic breakdown of dihydrouracil. Similarly, β-aminoisobutyric acid is formed by the hydrolytic breakdown of dihydrothymine. We found β-aminoisobutyric acid in

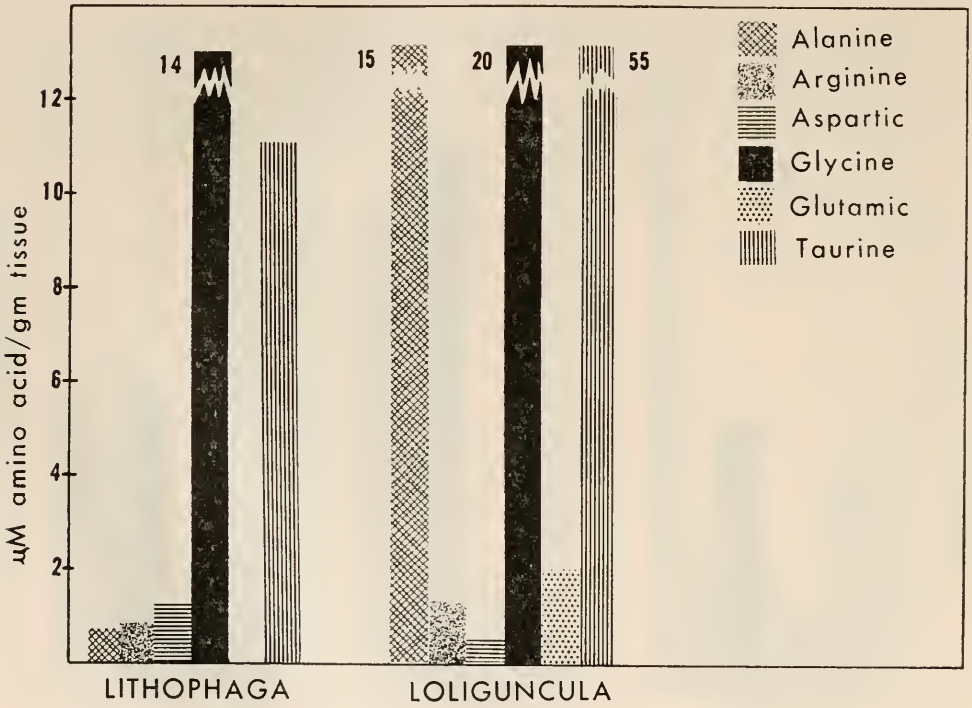

Figure 5. Estimated concentration of six amino acids in the tissues of pelecypods *Lithophage bisulcata* and the cephalopod *Loliguncula brevis*.

small amounts in *Volcella* and in larger amounts in *Mytilus*. A somewhat curious finding is that of asparagine in certain representatives of the Mollusca and Arthropoda, as shown in Table II. Asparagine is not often found in detectable amounts in mammals.

Arginine was present in all the species studied. It probably resulted from the hydrolysis of arginine phosphate, a well-known phosphagen in invertebrates.

Inasmuch as the taurine concentration appeared to vary with the environment we proceeded to study a number of fresh-water forms and also other marine forms for their taurine content. The results are shown in Table III. The presence of taurine by chromatography is detectable in concentrations as low as 0.1 micromole per gram. Our results indicate that taurine is either absent in the fresh-water forms of molluscs or it is present in concentrations lower than 0.1 micromole per gram. Every fresh-water and terrestrial mollusc studied showed no taurine whereas every form of invertebrate living in salt water or brackish water contained taurine in detectable and measurable amounts.

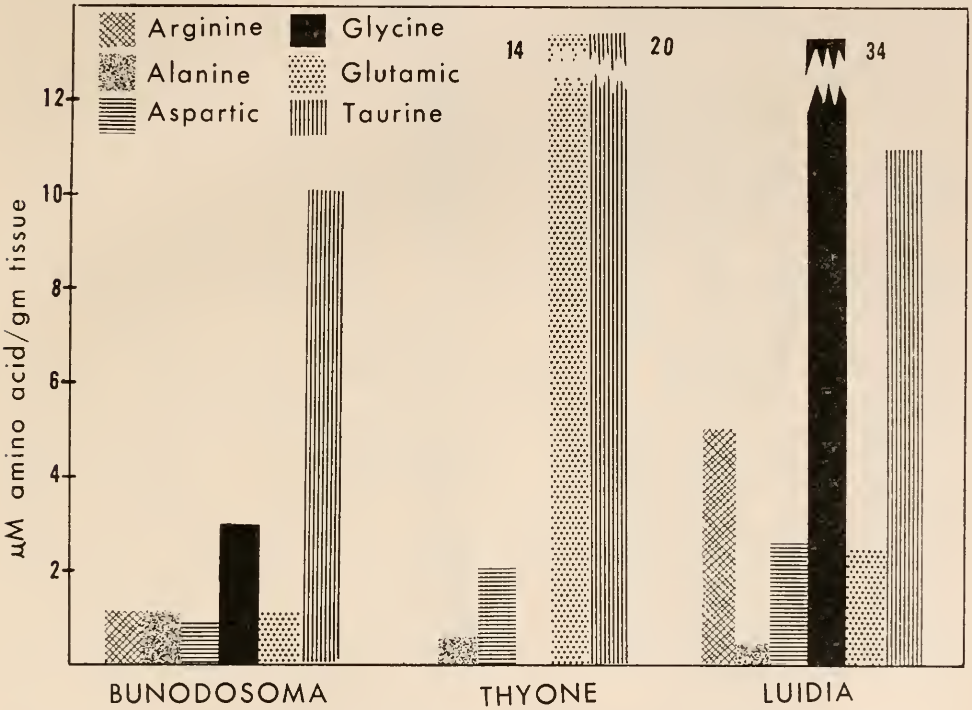

FIGURE 6. Estimated concentration of six amino acids in the tissues of the coelenterate *Bunodosoma cavernata* and the echinoderms *Thyone* sp. and *Luidia clathrata*.

At this stage of this study, it is not possible to attribute to taurine any definite role. But the evidence is suggestive that it plays a very important role as an osmoregulator.

TABLE III

Taurine content in various molluscs

Mollusca	Environment	Taurine
Gastropoda		
Lymnaea palustris	Fresh water	−
Marisa cornuarietis	Fresh water	−
Pomacea bridgesi	Fresh water	−
Rumina decollata	Terrestrial	−
Otala lactea	Terrestrial	−
Mesodon thyroidus	Terrestrial	−
Bulimulus alternatus	Terrestrial	−
Murex fulvescens	Marine	+
Littorina irrorata	Marine	+
Oliva sayana	Marine	+
Polinices duplicata	Marine	+
Busycon perversum	Marine	+
Siphonaria lineolata	Marine	+
Fasciolaria distans	Marine	+
Thais haemastoma haysae	Marine	+
Pelecypoda		
Anadonta grandis	Fresh water	−
Quadrula quadrula	Fresh water	−
Lampsilis sp.	Fresh water	−
Elliptio sp.	Fresh water	−
Rangia cuneata	Brackish-Fresh water	+
Brachiodontes recurvus	Brackish-Marine	+
Crassostrea virginica	Brackish-Marine	+
Donax variabilis	Marine	+
Venus mercenaria	Marine	+
Dosinia discus	Marine	+
Arca incongrua	Marine	+
Arca campechiensis	Marine	+
Noetia ponderosa	Marine	+
Cephalopoda		
Loliguncula brevis	Marine	+

SUMMARY

1. Free amino acids of 17 species of aquatic invertebrates were determined by chromatographic methods.

2. Qualitative differences in some amino acids were detected in organisms of different species. The concentration of alanine, arginine, aspartic acid, glutamic acid, glycine and taurine was measured and significant differences recorded.

3. Taurine was found in high concentration in all the marine organisms studied but was not found in several fresh-water and terrestrial organisms.

4. The possible role of taurine and other free amino acids in aquatic organisms is discussed.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1959

ABSTRACTS OF SEMINAR PAPERS

July 7, 1959

Isolation and chemical identification of the crystalline cytoplasmic inclusions in the large, free-living amebae. Joe L. Griffin.

The crystalline inclusions of *Amoeba proteus*, *Amoeba dubia*, and *Chaos chaos* have been shown to be composed of the same substance. This crystalline material has been isolated, purified, and shown to be identical with synthesized carbonyl diurea, with regard to physico-chemical properties, elemental analysis, x-ray diffraction patterns, infra-red spectra and optical properties. The two types of crystals found in the above species, plates and bipyramids, are apparently alternative crystalline arrangements of carbonyl diurea. It is suggested that carbonyl diurea represents an end product of nitrogen metabolism in amebae.

Physico-chemical characterization of chromatophorotropins in the crayfish Cambarellus shufeldti. Milton Fingerman.

Sinus glands and central nervous organs were observed with an electron microscope. These organs contained neurosecretory granules that were not bounded by a membrane. Organs containing these granules were extracted in hypotonic and hypertonic media. The hypertonic extract was more potent in concentrating red pigment. This result would not have occurred if the granules were bounded by a semipermeable membrane. Extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives were incubated with trypsin for one hour at 33° C. The chromatophorotropins were almost completely inactivated. Presumably the chromatophorotropins contain peptide bonds. These hormones are not inactivated by boiling. Extracts of the organs were then subjected to filter paper electrophoresis for two hours at pH 7.6–7.7. The dark red pigment dispersing hormone in the eyestalks and the concentrating hormone in the supraesophageal ganglia and circumesophageal connectives were electropositive. The dispersing substance in the latter two organs was electronegative. At pH 2.3 the dispersing hormone in the supraesophageal ganglia and circumesophageal connectives was electropositive; its charge had been reversed. The isoelectric point is probably about pH 4.0. When electrophoresis was performed at pH 8.9 some of the molecules of dispersing hormone in the eyestalk and of the concentrating hormone in the supraesophageal ganglia and circumesophageal connectives remained at the origin and some were electronegative. The isoelectric point of both substances is probably near pH 8.5. These chromatophorotropins are presumably polypeptides since their charge can be reversed, they are heat-stable, and they can be inactivated by trypsin.

This investigation was supported by Grant No. B-838 from the National Institutes of Health.

The molecular basis for the "catch" mechanism in molluscan muscles. William H. Johnson and Andrew G. Szent Györgyi.

It has been known for some time that certain muscles, such as the adductor muscles of bivalves, are capable of remaining contracted for considerable periods of time without much apparent utilization of energy. This paradox has been attributed by some to tetanic contractions of high efficiency; others have suggested that a change in the visco-elastic properties of the muscle occurs along with contraction which is capable of maintaining tension or shortening without requiring much energy. Evidence favoring the second type of mechanism has been obtained by one of us. Investigation of the properties of paramyosin, a protein found in large amounts in these muscles, has revealed a critical dependence of solubility upon the pH and ionic strength of the medium. Similar dependence on these parameters is seen when glycerinated fibers prepared from these muscles contract without load in ATP. These results suggested that paramyosin might be a key part of a system which determines the mechanical properties of the "catch muscles." To test this possibility, glycerinated fibers prepared from the anterior byssus retractor muscle of *Mytilus edulis*, a muscle which shows the above described behavior, were stretched and the tension thus developed measured under conditions where the actomyosin system should contribute little to the stiffness of the fiber. Salyrgan (10^{-4} *M*) and 10^{-2} *M* pyrophosphate were added to the medium. Measurements of fiber stiffness were made at various values of pH and ionic strength. At low ionic strength (0.07), fibers were relatively stiff at pH values below 6.5, a range in which paramyosin crystallizes out of solution, while at pH values above this, the fibers were relatively plastic. Thus the extensibility of the fibers parallels the solubility of paramyosin, and, since paramyosin is present in rodlets within the muscle, it might be thought of as a second system in parallel with the actomyosin system, which, when it is made stiff, is capable of maintaining tension initially developed by the latter system.

JULY 14, 1959

The physiology of the predator-prey relationship existing between Paramecium aurelia and Didinium nasutum. JAMES D. EISEN.

A study of the population interactions existing in a three-level food chain was made to determine the effect of providing various monofloral cultures of bacteria to the prey, *Paramecium aurelia* Müller and the subsequent effect of the paramecia, when used as food, on the reproductive rate of the predator, *Didinium nasutum* Müller.

Pedigree *P. aurelia*, variety 4, stock 51:7, sensitives, were transferred from a non-living, non-bacterial medium to five monofloral cultures of Gram-negative, non-spore-forming bacteria, and to a wild bacterial culture. The ciliates grew well on the wild bacteria and on all the monoflorae except one. From this it was concluded that mixed bacterial cultures were not essential for the satisfactory growth of *Paramecium*, but that not all monoflorae were nutritionally adequate. The growth-inducing powers of the bacterial species were found to be variable.

Clonal didinia were excysted at the beginning of each of the 16 five-day isolation-culture replications, sterilized, and placed along with 300 washed paramecia of each line into individual depression slides. Division rates of the predator on each *Paramecium* line were recorded for the first three days of each replication, and the means compared statistically. *Didinium* preying upon wild-bacteria-fed paramecia manifested a division rate of 4.9557 ± .0083; on the five monofloral-fed paramecia, division rates were .8510 ± .0520, 1.0942 ± .0552, 1.7721 ± .0388, 1.9700 ± .0237, 2.0527 ± .0343.

After several generations, all didinia except those on wild-bacteria-fed paramecia died. The statistically different division rates and subsequent death of the didinia appeared to be caused by nutritional deficiencies in the monofloral-fed-paramecia, possibly vitamins or amino acids, which were induced by the bacterial food ingested by the prey.

The inadequacy of paramecia fed on a monofloral culture of *Bacillus pyocyaneus* may have accounted for the death, rather than the encystment, of *Didinium* in Gause's classical predator-prey cycle. Gause's theory, that "immigrations" of both the predator and prey were necessary for the maintenance of the cycle, may not be wholly correct.

Life cycle and metabolism of a marine nematode, Enoplus communis. WOLFGANG WIESER.

The life history and metabolism of a marine nematode, *Enoplus communis* Bastian, was studied. The species has an annual life cycle, spawning taking place in early spring. Maturity is reached in fall and early winter, and both sexes over-winter with their gonads fully developed.

The metabolism is not temperature-compensated and there is no difference in respiration between the two sexes.

The respiration rate is fairly low in the fertilized, uncleaved eggs (about 600 mm.^{3} O_2/g./hr.). Right after hatching the oxygen consumption increases sharply to a value about three times that of the eggs. Then it drops quickly, but in animals weighing between 6 and 10 μg another sudden rise occurs, followed by a second drop and subsequent levelling of the curve. The first increase in metabolic rate from egg to first juvenile stage might be due to the activation of metabolically important substance. The following changes, however, are most likely correlated with the process of molting and are probably due to changes in the percentage of dry tissue matter in animals prior to and after ecdysis.

The large fluctuations in respiration rate occurring in animals of different size, despite a constant surface/volume ratio, suggest that body surface proper does not control oxygen consumption in this species. Rather, the relationship between size and metabolism has to be considered as a demonstration of the law of relative growth with respect to cells and cell constituents.

Uptake of strontium by Syracosphaera carterae. RALPH A. LEWIN.

Coccolithophores (Chrysophyta) frequently constitute the main component of marine phytoplankton, especially in warmer seas. Since their cells are characteristically invested in an armature of sculptured scales of calcium carbonate (coccoliths), they are evidently one of the chief biological mechanisms for calcium deposition in nature. The degree of strontium incorporation by coccolithophores is therefore of practical importance in relation to the rate of entry of Sr^{90} into the biosphere.

A synthetic sea-water medium was devised, containing 3.4 m*M* of Ca, pH 6.8. In replicate flasks, 0.0, 0.5, 1.0 and 2.0 m*M* of Sr were added. After autoclaving they were inoculated with a pure culture of *Syracosphaera carterae,* and were maintained at 22° C., aerated, and illuminated constantly at 2500 lux, for 25 days. The coccoliths were then harvested by differential centrifugation, washed, dried, and examined by x-ray diffraction (Dr. I. Fankuchen) and flame photometry (Dr. T. J. Chow).

In the absence of Sr, they consisted of pure calcite. In the presence of Sr, the incorporation or concentration factor (atoms Sr/Ca in coccoliths: Sr/Ca in medium) was approx. 0.02, indicating a high degree of discrimination against this element, under the defined experimental conditions. It is not known whether this selectivity is attributable entirely to chemical forces during crystallization of the calcite, or partly to biological specificity.

JULY 21, 1959

Motion pictures of mating behavior of Tetrahymena pyriformis rendered amicronucleate by x-ray treatments. CARL CASKEY SPEIDEL AND GEORGE GILBERT HOLZ, JR.

As previously reported by one of us (C. C. S.) for three strains of *Tetrahymena corlissi* the micronucleus, a genetic center rich in deoxyribonucleic acid, was eliminated by repeated severe x-ray treatments. Since mating in this species had not been observed, it became desirable to use another species in order to watch the mating behavior of amicronucleate tetrahymenae. This was done for axenic clonal cultures of *Tetrahymena pyriformis,* Mating Types I and II, Variety 1 (G. G. H.).

After receiving a cumulative dose of 2400 kr from four separate x-ray treatments of 600 kr each, the surviving progeny of Mating Type I consisted of amicronucleate individuals; those of Mating Type II consisted of both amicronucleate and micronucleate individuals. The mating behavior of the following combinations was observed and recorded by cinephotomicrography: (1) normal unirradiated tetrahymenae of Mating Types I and II, (2) x-rayed Types I and II, (3) normal Type I and x-rayed Type II, and (4) x-rayed Type

I and normal Type II. No difference was noted in the mating behavior of these four combinations. Conjugation of amicronucleate individuals of x-rayed Types I and II occurred. During conjugation of all four combinations, triples were occasionally formed. Descendants from a single mating pair of x-rayed Types I and II, kept in the same bacterized culture, still conjugated after six months. Likewise descendants from x-rayed Types I and II, kept in separate bacterized cultures for seven months, still conjugated. It is concluded that the presence of the micronucleus is not necessary for the mating reaction to occur.

This investigation was supported by a research grant (RG-4326 C2) to C. C. S. from the National Institutes of Health, Public Health Service.

Lysosomes and the physiology and pathology of cells. ALEX B. NOVIKOFF.

In 1956, with Beaufay and deDuve, we reported that electron microscopy revealed the presence in isolated lysosome fractions from rat liver of bodies resembling pericanalicular bodies seen *in situ*. However, the identification of the pericanalicular dense bodies as lysosomes was provisional since the fractions also contained a great many mitochondria. With Edward Essner, we have recently obtained support for this identification from an electron microscopic and cytochemical study of hepatocellular pigments in man. Lipofuscin, biliary pigment, iron, or the unknown pigment of chronic idiopathic jaundice may appear within the pericanalicular dense bodies, converting them into characteristic pigment granules. The granules retain acid phosphatase activity. Parallel electron microscopic and staining studies of liver, kidney, and macrophages show that the Gomori staining reaction for acid phosphatase activity, when performed on frozen sections (or smears) of cold formal-calcium-fixed tissues (or cells), is a reliable means of visualizing lysosomes. Other studies support deDuve's recent suggestion that lysosomes play important roles in pinocytosis, phagocytosis and lytic processes. Among the material studied are pinocytosis vacuoles of *Chaos chaos;* digestive vacuoles of *Ameba proteus;* phagocytosis in erythrophagocytes and in macrophages of spleen, liver, lung and other tissues of rat, mouse, and man; many normal tissues; rat kidney following intraperitoneal protein injection; cytolyzing cells in normal physiological or developmental circumstances (atretic ovarian follicles, resorbing tadpole tails, regressing chick Mullerian ducts); dying cells, as in keratinization of squamous epithelia, and pathological situations (following ligation of vasculature to liver or kidney, or of bile duct, or of ureter; or during feeding of carcinogenic azo dyes.) From our survey we conclude that most bodies described by electron microscopists as "microbodies," "cytosomes," and "large granules" are lysosomes—cytoplasmic organelles delimited by "single" outer membranes and possessing high levels of acid phosphatase and other hydrolases with acid pH optima.

JULY 28, 1959

On the occurrence of myoglobin and cytochrome oxidase activity in the cartilaginous odontophore of Busycon canaliculatum. PHILIP PERSON, JAMES W. LASH AND ALBERT S. FINE.

Both myoglobin and cytochrome oxidase activity have been identified in the above tissue. Aqueous solutions of the oxidized myoglobin possess α, β and γ peaks at 574-575, 538-539 and 415-416 mμ, respectively. In the dithionite reduced state, the α and β peaks are replaced by a lower, single broad hump, between 540-565 mμ. The soret absorption decreases and moves to 430 mμ. The pyridine hemochrome prepared from the myoglobin has an α peak at 554-555 mμ, a β peak at 524-525 mμ, and a third, smaller peak at 480 mμ, in the visible spectrum. The soret absorption of the hemochrome is at 417-418 mμ. Washed particulates prepared from homogenates of the tissue could oxidize reduced cytochrome *c*, as demonstrated in spectrophotometric experiments. The loss in absorbance of reduced cytochrome *c* at 550 mμ was -0.264/3 minute interval/mgm. dry weight. In a manometric assay for cytochrome oxidase activity using hydroquinone as substrate and added cytochrome *c*, the Q_{O_2} (dry weight) was 9. This is the first reported occurrence of myoglobin and cytochrome oxidase activity, together, in a cartilage type tissue.

Electron microscope study of the sperm of Crassostrea virginica. PAUL S. GALTSOFF AND D. E. PHILPOTT.

Mature spermatozoon of the oyster examined under highest magnification available with phase contrast microscope was observed to contain a light refracting body at the center of the nucleus under the acrosome. This observation led to a detailed study of the sperm by means of electron microscopy. Sperm balls removed from the tubules of the testis were fixed in buffered 1% osmic acid, embedded in plastic, and sectioned. The structure of the spermatozoon was reconstructed from over 300 photographs taken at various magnifications and at different planes. The tip of the slightly granular and homogeneous nucleus is covered by a conical acrosome of highly osmiophilic substance. The center of the nucleus is occupied by an egg-shaped body which extends from the apex of the acrosome almost to the base of the nucleus. This structure is undoubtedly a permanent feature of the spermatozoon and is probably related to the formation of the axial filament. It has been named by us the "axial body." The core of the axial body consists of a central stem of slightly denser material.

The centriole, at the base of the nucleus, is a hollow, cylindrical body measuring about $0.2\,\mu$ in length and 0.1–$0.18\,\mu$ in width. The centriole walls are constricted into several sections of a material of alternating density. Nine bands of denser material are clearly seen at the cross-section of the centriole. The lower part of the centriole is in close contact but separated from the basal plate of the tail. Four mitochondrial bodies surround the centriole and are connected to its wall. The structure of the tail is similar to that of a cilium; it consists of a pair of axial filaments surrounded by a ring of nine double filaments emerging from the basal plate. The pair of axial filaments does not originate from the basal plate and consequently the proximal part of the tail consists only of nine peripheral filaments. The peripheral filaments of the tail are connected to the outer walls and to the axial filaments by radial trabeculae.

AUGUST 4, 1959

Some structures found in electron microscopic studies of an amphibian tumour. VINCENZO LEONE.

In the course of an electron microscopic study of a tumour (reticulo-lympho-sarcoma) induced by injecting methylcholanthrene into the newt, *Triturus cristatus* L., unusual structures were found in some of the cells, in the spleen.

These studies have been carried out in the Dept. of Zoology, University of Milano, Italy, using a Philips 75 KV electron microscope, and at the Institute du Cancer Gustave Roussy, Villejuif, France, using a Siemans electron microscope: Elmiskop I. Large areas of the cytoplasm containing masses of dense material, bounded by a limiting membrane and containing granules within vacuoles, were seen between the more or less altered organelles of the cytoplasm. Similar masses containing vesicles and large granules were likewise observed. The latter were usually more dense, and showed an ill-defined internal structure. It is possible that the two types of formations described are similar reaction bodies in different stages of evolution. In some cases, cells in both the spleen and the liver were crowded with bacteria and they showed a typical reaction; the cytoplasm contained large, round dense bodies surrounded by membranes. At times they contained vacuoles of various sizes and shapes. Although in most cases the cytoplasmic reaction masses surround and engulf bacteria, these masses are not always related to the bacterial bodies. Frequently the reaction phenomenon is represented by a clearly visible series of concentric membranes. The bacterial bodies within the masses give the appearance of being somewhat digested. Osmiophilic bodies and (empty?) vacuoles are also frequently found. In some cases numerous bacterial bodies, surrounded by one or two membranes, are found in the reaction zones; in others only one or two bacteria are found within the reaction zone.

I would like to emphasize that this amphibian material illustrates a very spectacular reaction to a living (bacterial) agent. This agent produces a very pronounced but not a lethal influence. The observed association of cell (macrophage) + bacteria (non-pathogenic) gives a clearer picture of a process (phagocytosis?) than is the case with most of the agents usually considered as responsible for the induction of inclusion and reaction bodies.

Electron microscope study of the integument, flame cells and gut of the human parasite, Schistosoma mansoni. ALFRED W. SENFT.

An electron microscope study of the trematode, *Schistosoma mansoni*, was undertaken in order to demonstrate the basic structure of certain portions of this blood parasite. It was hoped that clues concerning the physiology of the integument, flame cell structures, and gut might be deduced from the ultrastructure morphology.

The integument was found to consist of a multivacuolated, highly frondular surface, penetrated at intervals by spines. The presence of an enormous number of "Swiss cheese" lacunae in the outermost layer suggests that the integument is a huge absorbing surface which transports the major portion of the nutrients required by the schistosome. A thin osmophobic membrane is immediately subjacent to the outer surface. The spines appear to be imbedded in this clear membrane. Muscle layers, both horizontal and circular in arrangement, were seen beneath the osmophobic layer. Small canaliculi proceeding from deeper body areas and penetrating the integument were suggested in some views, although one could not be certain that these were not artifacts.

The flame cells were found to consist of bundles of about 80 cilia. These are enclosed by a single or double layered membrane. The membrane itself has a series of stiffening members which are arranged in a stockade fashion around the bundle of cilia. It is postulated that this membrane is freely permeable to body fluids. The stiffening members probably keep the lumen of the flame cell patent in the presence of variable pressures.

Cilia were noted to have two or four central fibrils, and nine pairs of fibrils arranged concentrically around the center fibrils. Distal to the flame cells, collecting tubules were shown to have lamellated membranes. These might function as check valves, preventing reverse flow of fluid which the flame cells put in motion.

The gut was shown to have an extensive fibrillar or microvillar surface.

Fine structure of skeletal muscle from Limulus polyphemus. G. W. DE VILLAFRANCA AND D. E. PHILPOTT.

Observations of *Limulus* skeletal muscle with both phase contrast and electron microscopes reveal sarcomeres, about 7.5 μ at rest length, which are cross-striated. The dark A band (5.0 μ) is of uniform density: no H or M bands are present. The I band (2.5 μ) is bisected by a dark Z band which, in some sections, seems to be continuous across the whole fiber and terminates on the sarcolemma in a plate-like structure. There is, however, complete separation by a membrane of the sarcolemma and Z band. In other sections the Z band is interrupted by vesicular material between two mitochondria. Mitochondria are relatively rare and occur on both sides of the Z band region, adjacent to the I band. Extensive vesicular material, "sarcoplasmic reticulum," occurs at the level of the A band, encircling the myofibrils, and connecting with the cell surface at the Z band level.

The A band consists of large filaments about 140 Å in diameter and spaced from 170 to 200 Å apart. These filaments run continuously through the A band, parallel to the major axis, and are of constant diameter. Apparently the large filaments are interconnected by small (30 Å), filamentous "cross-bridges" which, in some cases, appear to be coiled around the large filaments. In electron micrographs of very thin sections (about 1 filament thick) no "secondary" filaments are observed between the large "primary" filaments; nor are any observed in sections where large filaments run in and out of the plane of section. In cross-section, one may observe an hexagonal pattern of large filaments with cross-bridging filaments between and at right angles to the large filaments.

On the basis of these observations, horseshoe crab muscle could not contract by a sliding filament mechanism as proposed for other muscle by Huxley, but, as in vertebrate smooth muscle, must contain a contractile system within a single set of filaments.

AUGUST 11, 1959

Present status of the problem of plasmalogen structure. MAURICE M. RAPPORT.

Plasmalogens are glycerophosphatides that release higher fatty aldehydes under hydrolytic conditions. The organic radical responsible for this reaction was recently shown to be an

α, β-unsaturated ether. It is unique among natural products: carbon in the aldehyde oxidation state participates in double bond formation (hydration of this bond, followed by hydrolysis, generates aldehyde). This double bond, much more reactive than the normal type, gives rise to several remarkable addition reactions.

Pure native plasmalogens are not yet obtainable. However, a pure, crystalline derivative, known for many years, has been assigned an acetal structure. This was shown to be incorrect, and the true α, β-unsaturated ether structure was first conclusively proved, using crystalline preparations from both ox muscle and brain. This proof was based on the demonstration that hydrogen addition rather than hydrogenolysis produced the loss of aldehydic reactions attending hydrogenation. Confirmation was obtained by means of a reaction specific for the "activated" unsaturation in α, β-unsaturated (vinyl or enol) ethers: addition of iodine in aqueous methanol. Under conditions where such ethers add 95% of the stoichiometric quantity of iodine, no reaction is given by ordinary double bonds, aldehydes, acetals, hemiacetals, or acetylenes.

Availability of two independent, stoichiometric, analytical methods presented a rare opportunity for examining the total plasmalogen content of different tissues. Thus, the correlation of iodine uptake and aldehydogenic content (based on p-nitrophenyl-hydrazone formation) for total lipid extracts of tissues from rabbit, rat, and man showed that almost all mammalian plasmalogens must be α, β-unsaturated ethers.

Unsolved structural problems are three: 1) is the aldehydogenic chain connected to the primary or secondary hydroxyl group of glycerol (the remaining hydroxyl group is esterified to fatty acid); 2) are some plasmalogens not phosphatides (reported for Asterias); and 3) do true acetal plasmalogens occur naturally (reported for Anthopleura)?

The incorporation of radioactive label into RNA: synthesis or terminal addition. W. S. VINCENT AND ELYANE BALTUS.

An analysis of the specific activities of individual nucleotides from the RNA of starfish oocyte cytoplasm after exposure to P^{32} has revealed striking inequalities in the labelling of specific nucleotides. Such inequalities suggested that terminal addition of nucleotides to a pre-existing RNA molecule was taking place, rather than synthesis of new higher polynucleotides.

At six hours the relative specific activity of nucleotides isolated from KOH hydrolysates was Cy: 41; Ad: 42; Gu: 10; Ur: 6. Such data would be consistent with the hypothesis that two cytidylic acid molecules were added to an RNA molecule terminating with an adenylic acid residue. Partial confirmation of this hypothesis is found in shorter time experiments, where after two-hour incubation the following relative specific activities were determined: Cy: 20; Ad: 60; Gu: 14; Ur: 6. Analysis of a diesterase digest revealed the following distribution of label: Cy: 46; Ad: 1; Gu: 0; Ur: 53.

On the basis of these data it is likely that most of the radioactive label incorporated into the starfish cytoplasm RNA occurs as the addition of two cytidylic and a terminal uridylic acid residue to an adenylic acid residue present on a pre-existing RNA molecule.

On the basis of the experiments cited, we conclude that a precise analysis of the distribution of label in the RNA molecule is necessary in order to determine whether or not net synthesis has occurred. Results from autoradiographic techniques become particularly difficult to assess, for they are not susceptible to rigid biochemical control.

AUGUST 18, 1959

The action of glycerol on protoplasm. L. V. HEILBRUNN.

Various authors have shown that when some types of cells are previously treated with 10–30% solutions of glycerol, these glycerinated cells become extremely resistant to very low temperatures. In muscle physiology, treatment of muscle fibers with 50% glycerol has become very common practice. And yet there has been but little effort to determine what the glycerol does to the physical state of the cells which have been bathed in it.

Unfertilized eggs of Arbacia and Chaetopterus and fertilized eggs of the clam Spisula were exposed to 5%, 10%, 15% and 20% glycerol in sea water and the viscosity of the cytoplasm was then determined by the centrifuge method. Even in the 5% solution there is a large increase in viscosity; higher concentrations cause an increase in viscosity so great that it can no longer be measured. Muscle fibers were exposed to 50% glycerol and were then injected with isotonic KCl solution. If such a solution is injected into fresh muscle fibers, it spreads rapidly through the fiber—also, when the micropipette is removed there is no trace of where the pipette had been. However, in the glycerinated fibers the injected fluid remains as a discrete droplet; and when the micropipette is removed, the hole it had caused in the fiber remains clearly evident. It is as though a sharp instrument had been plunged into a piece of cheese.

Obviously, both in marine eggs and in muscle fibers, glycerol can cause a gelation of the protoplasm. Thus one can understand the great resistance of some glycerinated cells to low temperatures; for ice crystals, which can readily form in protein sols, can not form in gels. Also, in the case of muscle, contraction of a glycerinated muscle fiber involves some change in a gel, whereas the normal muscle fiber is largely fluid.

Aided by a grant from the National Science Foundation.

Electrophoretic studies on protoplasm. WALTER L. WILSON AND K. S. SWAMI.

Observations were made on unfertilized marine eggs subjected to an electrical current to determine whether the cytoplasmic inclusions move to the cathode, in which case they bear a positive charge, or to the anode, in which case they bear a negative charge. The eggs of Arbacia, Asterias, and Echinarachnius in sea water undergo cytolysis when subjected to an electrical current. This reaction does not occur in the absence of calcium ion. Thus, eggs in sodium citrate (0.35 *M*) do not undergo cytolysis during the flow of an electrical current. Under these conditions granules move toward the cathode and in almost all cases in Arbacia and Asterias some granules move toward the anode, leaving a clear area on the cathodal side of the egg. However, the clear area on the cathodal side of the egg is much smaller than that on the anodal side. In the egg of Chaetopterus in sea water there is a breakdown of cortical granules and a movement of granules toward the cathode, but if the current intensity is increased the whole mass of protoplasm seems to coagulate and moves toward the anode. The egg of Hydroides in sea water, or in sea water plus citrate, reacts like the eggs of Arbacia and Asterias in citrate. In the egg of Spisula in sea water there is a movement of granules and nucleus toward the cathode, but only after marked swelling of the egg. If these eggs are immersed in citrate for short periods there is a movement of granules toward the anode, but after about an hour the granules move toward the cathode.

This work was supported by a grant from the National Science Foundation, administered by Walter L. Wilson.

Germinal vesicle breakdown in the eggs of Spisula and Hydroides. FRANCIS T. ASHTON.

Germinal vesicle breakdown, an easily observable example of the first step in typical cell division, can be induced experimentally by many chemical and physical agents. These agents act either on the cortex, where they release calcium, which activates a protease system, or directly on the nuclear membrane. Allen (1953) showed that many agents, among them excess isotonic potassium and hypertonic solutions, act on the *Spisula* egg only in the presence of calcium. In addition, I have found that chymotrypsin will act in the absence of calcium. *Hydroides* eggs may be activated in the above ways, by heat and cold, and by trypsin in the absence of calcium.

If, on activation, calcium releases a protease, then enzyme inhibitors as well as calcium-binding agents should affect activation. Soy bean (trypsin) inhibitor and Ovomucoid delay but do not prevent germinal vesicle breakdown in *Spisula.* Iodoacetamide delays the breakdown and markedly reduces the number of eggs activated.

If *Spisula* eggs are homogenized in sea water and the homogenate centrifuged, the supernatant contains a substance which will activate fresh *Spisula* eggs. This activity disappears on the addition of soy bean inhibitor, and is reduced by heating. Homogenates made in citrate do not show the activity.

When *Spisula* eggs are activated with excess KCl in sea water and homogenized in citrated sea water at the time of nuclear breakdown, the supernatant contains an activating substance. The activity disappears on the addition of Ovomucoid or iodoacetamide. The peak of activity occurs at the time the germinal vesicles are breaking down, not earlier or later. The substance extracted from the homogenates is undoubtedly a calcium-activated protease, requiring SH groups. It is also the enzyme responsible for the breaking down of the germinal vesicle.

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ELECTROBIOLOGY SEMINARS

July 20, 1959

The fine structure of synapses. A. J. de Lorenzo.

Examination of numerous synaptic junctions with the electron microscope has revealed a remarkable uniformity in fine structure, *i.e.*, most synapses are characterized by (a) closely applied limiting membranes of the synaptic processes separated by a cleft about 200 to 300 Å wide, with occasional areas of increased density; (b) presynaptic terminals containing mitochondria and clusters of "synaptic vesicles."

Utilizing this fine-structure model, smooth muscle of the iris and small intestine were examined with particular reference to innervation. Nerve endings in iris muscle are numerous and contain large numbers of vesicles. Synaptic membranes are separated by a cleft 75 to 100 Å wide. Individual muscle cells contain little endoplasmic reticulum and are separated by large intercellular spaces. On the other hand, intestinal muscle cells contain a paucity of recognizable nerve endings. Synaptic clefts are 75 Å wide and endoplasmic reticulum is more abundant in the muscle cells.

Since it has been suggested that "synaptic vesicles" which occur usually in presynaptic processes may be involved in chemical transmission, certain electrical synapses in the crayfish were also studied. Examination of the crayfish lateral giant-to-motor synapse reveals motor fiber processes terminating on the lateral giant pre-fiber in very close contiguity. A synaptic cleft about 75 Å wide separates the synaptic membranes. Curiously, only the *post-synaptic* processes contain clusters of vesicles and the presynaptic fiber demonstrates no structural specialization. Cholinesterase staining of this synapse was routinely negative.

These observations suggest that fine-structure differences exist at smooth muscle endings and in electrical synapses of the crayfish when compared with the classical terminal bouton. It would seem wise, however, to approach with caution any interpretation of the nature of synapses based upon dimensions of synaptic clefts and locations of "synaptic vesicles," until more evidence is forthcoming. Possible role of "synaptic vesicles" at electrical synapses remains unresolved.

Presynaptic activity in drug-induced neuromuscular facilitation. A. S. Kuperman, G. Werner and E. W. Gill.

Almost 60 compounds were tested for their capacity to facilitate neuromuscular transmission in the cat, and those found effective are divisible into two groups. In one group the dose-response regression lines for twitch potentiation are parallel and steep, and significant correlation exists between facilitating potency and anti-ChE activity. In the other group dose-response lines are parallel but flat, and there is no relationship to ChE inhibition. The structural requisites for twitch potentiation and curare antagonism are also clearly different for each group. Although this evidence indicates a functional distinction between the two classes, other evidence reveals a common presynaptic mode of action for all facilitating drugs. All agents produce post-activation repetitive discharge of motor nerve which originates from the axon terminals. Furthermore, this nerve terminal action, twitch potentiation, and curare antagonism are enhanced by tetanic nerve stimulation, double volleys, and subfacilitating doses of physostigmine; these effects are suppressed by anesthetic doses of pentobarbital and subparalytic doses of curare. Each of these modifications of drug-induced facilitation was shown to act exclusively through a presynaptic mechanism.

Accordingly, it is proposed that all facilitating agents have a common presynaptic action; superimposed on this, in consequence of a specific molecular configuration, is another action which bears relation to anti-ChE activity. In these latter molecules, the contribution of presynaptic and anti-ChE effects to facilitation cannot be separately evaluated. Therefore, since these agents have a nerve terminal action which is greater than that induced by agents having only the presynaptic effect, the anti-ChE correlation may require explanation within a context other than the ACh-ChE theory of transmission.

The presynaptic events capable of being altered by drugs were found to be in the nature of nerve after-potentials. Recognition of these neuronal events which precede the transmitter process and are susceptible to distinct influences by chemical agents is necessary to the proper interpretation of pharmacologic studies; its significance to the physiology of mammalian neuromuscular transmission remains to be evaluated.

JULY 27, 1959

The chemistry of ion transport and its relation to excitation in nerve and muscle. BENJAMIN LOWENHAUPT.

Consideration of the answers to two questions has suggested a possible molecular model for excitation:

I. What may be known about the chemistry of ion transport?

A molecular model for transport, initially developed to explain transport in aquatic plants, is compatible with many biological materials. Its features include the following:

1. There are carrier molecules which have more or less specific chemical affinity for the transported ion and which successively bind ions from the ambient solution and release them back into solution.
2. The reactions for binding and releasing the ions are redox reactions of the carriers, *i.e.*, they constitute a redox pump.

II. Do chemical reactions for transport also occur for excitation and, if so, can insight into the chemistry of excitation be drawn from what is understood about the chemistry of transport?

Affirmative answers to this question indicate that excitation is a sudden oxidation of carrier molecules with concomitant release of ions, a wave of oxidation propagated along the cell. The name, *oxidation-wave hypothesis,* refers to this molecular model of excitation. It is supported by the following arguments:

1. A redox pump occurs in excitable cells, as in cells and tissues which transport. Furthermore, its activity parallels the rate of the bioelectric changes of excitation.
2. The kinetics of excitation appear the same as the kinetics of reactions for binding and releasing ions, the same reactions as for transport.
3. A sudden oxidation does occur during excitation.
4. If oxidizing muscle during excitation causes it to contract, the coupling between muscle excitation and contraction is established. Experimental observation is that oxidized muscle does contract, whereas it relaxes when reduced.

AUGUST 3, 1959

Effects of estrogen and progesterone on single uterine muscle fibers in the rat. J. M. MARSHALL.

White rats were ovariectomized and treated as follows: group 1, 6.0 μg. estradiol benzoate daily for five days; group 2, 6.0 μg. estradiol for three days, then 1.6 μg. estradiol plus 12 mg. progesterone for five days; group 3, untreated animals. Membrane potentials were recorded from single uterine fibers, and the tension was recorded from the entire uterine horn.

The untreated uterine fibers were quiescent, having a mean resting potential of 35.2 mv. Fibers from the estrogen-dominated uteri were rhythmically contractile and had a mean resting potential of 57.6 mv. A train of action potentials accompanied and preceded each contraction of the muscle. In certain areas the fibers showed pacemaker-like characteristics, *i.e.*, slow membrane depolarization between action potentials. Progesterone-dominated fibers had significantly higher resting potentials, mean 63.8 mv., but no localized pacemaker areas. Action potentials did not consistently precede or accompany contractions.

In groups 1 and 2, acetylcholine stimulated contractions, lowered the membrane potential and increased the discharge rate of action potentials. Epinephrine diminished contractions, raised the membrane potential and abolished the action potential discharge. The untreated uteri were unresponsive to both of these substances.

GENERAL SCIENTIFIC MEETINGS

AUGUST 24–27, 1959

Abstracts in this section (including those of Lalor Fellowship reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Contraction of muscle induced by direct light stimulation. PHILIP B. ARMSTRONG.

It has long been known that contraction can be induced by light stimulation in certain muscles without the mediation of nerves. The classic examples are the sphincter pupillae muscles of some of the fishes and many of the amphibians. Among the fishes the sphincter pupillae of the eel serves as a particularly good preparation for experimental study.

After excision of the eye the cornea is dissected off, the posterior three-fourths of the eyeball and the lens removed, and the remaining part of the eye, which includes the iris, is pinned out in Ringer's solution on beeswax with the outer surface up. The preparation is mounted on the stage of the microscope, a substage lamp is used and changes in pupillary diameter are measured with a micrometer in the ocular of the microscope. Light from the substage lamp does not reach the sphincter pupilla because of the heavy melanin pigment on the posterior surface of the iris.

If the preparation is placed in the dark, adaptation takes place with the pupil becoming fully dilated within 20 minutes. If light is then thrown from above on what is the front of the iris, constriction follows which comes on after a short refractory period and is complete within 30 seconds with light of adequate intensity. Graded responses are obtained with properly selected intensities of light. Adaptation of the sphincter pupillae to light is striking. If the pupil is maximally constricted by light and the illumination of the iris continued at this intensity, adaptation takes place with a dilation of the pupil to about 35% of the initial maximal constriction in 35–40 minutes. With submaximal light constriction adaptation also takes place. However, increased light intensity will induce constriction from the light adapted condition.

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Inhibition of fertilization and agglutination in Arbacia by an extract of Fucus. JOSEPH M. BRANHAM AND CHARLES B. METZ.

Investigations in Runnström's laboratory have shown that certain extracts of the brown alga *Fucus vesiculosus* inhibit fertilization in sea urchins. It seemed of interest to examine the local material for similar effect and to investigate further the mode of action of the inhibitor(s).

Extracts which inhibited fertilization in Arbacia were obtained by repeated extraction of fresh *Fucus vesiculosus* in distilled water at room temperature. The combined extracts were concentrated under vacuum at 50° C. and ten volumes of 95% ethanol added. A precipitate was discarded and the ethanol distilled from the soluble fraction under vacuum. This concentrated solution was finally dialyzed against sea water. Such solutions inhibited fertilization in dilutions exceeding 6×10^{-3}. Fertilization inhibition did not result from an irreversible action on the sperm, for sperm washed from the extracts showed undiminished fertilizing capacity. The *Fucus* extract appears to inhibit eggs irreversibly, for washing did not restore fertilizability to treated eggs. However, even following prolonged treatment in the extracts fertilized eggs were capable of some development.

The *Fucus* extract not only inhibited fertilization but also the agglutination of sperm by fertilizin. This action did not result from an irreversible interaction of the extracts with fertilizin since treatment with activated charcoal restored agglutinating action to non-agglutinating inhibitor-fertilizin mixtures. The agglutination inhibiting action appeared to result from a blocking of the surface of the sperm. Sperm washed from the extracts were not agglutinated by fertilizin. Moreover, the inhibiting effect was reduced or removed from the extract by sperm. Furthermore, sperm treated with the *Fucus* extract did not reduce the agglutinating power of fertilizin as did control sperm. Thus it seems that the *Fucus* extract reacts irreversibly with the antifertilizin on the sperm to render it unreactive to fertilizin. However, such blocking does not diminish the fertilizing capacity of the sperm.

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Central nervous aspects of firefly excitation. JAMES F. CASE AND JOHN BUCK.

We previously reported the occurrence, in the posterior abdominal nerve cord, of characteristic volleys of nerve impulses which precede each voluntary flash. Subsequent volleys have now been recorded from the subcuticular surface of the lantern and have been shown to precede induced as well as spontaneous flashes. Although double volleys initiate double flashes, and volleys recorded from widely separated regions of the lantern may differ slightly, there is little apparent correlation between volley duration and impulse frequency and the intensity of the succeeding flash or area of photogenic tissue involved. This suggests either that there is further, as yet undiscovered, neural modulation or that the tissue has considerable effector lability. The possibility of secondary modulation is perhaps supported by the fact that although flashes are always preceded by volleys, not all volleys eventuate in flashes. Furthermore, some individual fireflies are quite refractory to electrical stimulation, or even to the induction of pseudoflashes after hypoxia, until aroused. This inactive state may be associated with the persistent endogenous diurnal periodicity of spontaneous flashing activity described some years ago. Second order excitation could involve either a separate, more profuse peripheral nervous network, or a state of subthreshold facilitation maintained by "trophic" central activity correlated with behavioral state. There is considerable neural activity between volleys recorded from the cord, and some single spikes are seen between volleys in the lantern, but seemingly not enough to account for maintained priming. However, additional indications of central involvement are seen in the facts (a) that both voluntary and induced flashes may be completely inhibited by optic nerve stimulation, and (b) that eserine induces, in lanterns with central connections but not in denervated ones, asynchronous unit effector activity which may go through regular cycles of block and recovery.

Purification and properties of a ribonuclease from squid. MARY EDMONDS AND JAY S. ROTH.

The isolation and comparative study of ribonucleases differing from bovine pancreatic ribonuclease is of value for determining the relation of protein structure to enzyme activity, since the structure of the bovine enzyme is well known. Ribonucleases of known specificity are also useful for investigations of polynucleotide structure.

Squid gill and caecal fluid were examined. The former contains modest amounts of an acid ribonuclease associated with a particulate fraction that sediments at 10,000 × g. Caecal fluid contains large amounts of a soluble acid enzyme and for this reason was studied extensively. The caecal enzyme has an optimum pH at 5.3, is not affected by sulfhydryl reagents and is stable to heating at 60° C. for 5 minutes. It precipitates at 80% saturation with ammonium sulfate and is stable to treatment with acid. Thus, except for the pH optimum, it resembles crystalline bovine pancreatic ribonuclease in some respects. The squid caecal enzyme was purified approximately 300-fold by heat treatment, absorption of impurities by DEAE cellulose and, finally, salt gradient elution from a carboxymethyl cellulose column. The purified ribonuclease was free of phosphodiesterase and phosphatase activity. It split both polyadenylic and polyuridylic acids but did not appear to hydrolyze cyclic uridylic-, cyclic cytidylic-, or cyclic adenylic-3′,5′-phosphates appreciably. It also did not hydrolyze adenosyl-3′- or 5′-benzyl phosphates and, therefore, may require a purine-purine, purine-pyrimidine or pyrimidine-pyrimidine diester phosphate linkage for activity.

(Supported by grants from the National Cancer Institute and National Science Foundation.)

Isolation and characterization of cortical granule-hyaline material of the Arbacia egg. R. G. Faust, R. F. Jones and A. K. Parpart.

The material released from the cortical granules of the egg of *Arbacia punctulata* upon fertilization appears to be a mucoprotein. It has been isolated by allowing de-jellied, unfertilized *Arbacia* eggs to settle from sea water suspended above a 20–80 mixture of isosmotic sucrose and glycerol. During the settling the cortical granules explode and release their contents which is soluble in the non-electrolyte. Light centrifugation, removal of the supernatant sea water and collection of the egg-free sucrose-glycerol mixture gives a solution which, when brought to pH 2 to 3 with HCl, forms a white precipitate. This precipitate is washed 4 times with H_2O-HCl at pH 2–3, packed centrifugally and frozen dried.

The mucoprotein thus obtained contains 11% nitrogen, *ca.* 2% hexoseamine, 28% polysaccharide and 70% protein. Glucosamine and galactosamine are the amines, while galactose, fucose and mannose are present in descending concentration. No ribose is observable. The protein contains 17 amino acids. Analytical centrifuge data indicate a single molecular species of about 100,000 M.W.

This cortical granule mucoprotein is digested by α-amylase, but not affected by β-amylase, hyaluronidase, lipase, trypsin or papain (at pH 6); nor is it affected by pepsin (at pH 2). In all of these respects it resembles the hyaline layer of the fertilized egg. Both the cortical granule mucoprotein and the hyaline are depolymerized in the presence of non-electrolyte and both are repolymerized when Ca or Mg are added. Both show marked contraction at pH 2 to 3 and reversal at pH 7 to 8.

Cation exchange in the erythrocytes of the mackerel, Scomber scombrus. James W. Green, George Holsten and T. A. Borgese.

Red cells of fresh mackerel blood, obtained from pooled samples from a number of fish by heart puncture, were washed twice in 0.26 *M* NaCl and incubated at 15° or 25° C. in this medium in 10–20% cell suspensions in the presence of trace amounts of Na^{24} or K^{42} and 5–10 mM/l. of glucose. Influx or efflux of Na or K was measured over a two-hour period during which the cell concentrations of Na and K remained essentially stable at a level of 77.3 and 130.2 mM/l. cells, respectively. In the absence of information to the contrary the ions were treated as being freely exchangeable and their movements as occurring in a steady-state, two-compartment system. K flux was found to average 20.2 and Na, 50.9 mM/l. cells/hr. at 25° C. Some variability in the exchange rates from experiment to experiment was found. These exchange rates are higher than have been reported for man, beef or duck red cells and are higher than human red cells even when a comparison is made between the exchange rates per unit surface area of red cell. These measurements are interpreted to mean that the penetration rate of Na and K in mackerel red cells is greater than similar rates previously reported for other species, and indicate either a higher metabolic rate or the use of a larger fraction of metabolic energy for the maintenance of ionic equilibria than is found in these other species.

Movements of the cell membrane of some fresh- and salt-water amebae. J. L. Griffin and R. D. Allen.

The behavior of the cell membrane of amebae during locomotion has been an important consideration in some theories of ameboid movement. The surface tension theory became untenable when Jennings showed that the membrane moved forward as the ameba moved and not backward as the theory demanded. It has been assumed in some more recent considerations of ameboid movement (Goldacre and Lorch) that the membrane is formed at the front of the cell and destroyed at the rear. This assumption is not supported by the evidence reported in the past by Jennings, Dellinger, Schaeffer, and especially Mast, who observed the behavior of various inert particles attached to the surface of moving cells.

We have made further observations on the behavior of such inert particles (carmine, Chinese ink) attached to the surface of *Amoeba proteus, A. dubia, A. discoides, Chaos chaos, Pelomyxa palustris,* and several small species of marine and fresh-water amebae. In amebae exhibiting "walking movements" and in those attached near the tail, particles attached to the plasmalemma move forward with the ameba. In amebae which are firmly attached to the substratum at the front, the membrane and attached particles roll forward over the ameba as the specimen advances. The plasmalemma is not formed and destroyed every time the organism moves its own length. Instead, as Mast showed, the cell membrane slides freely over the hyaline ectoplasm except at those places where the ameba is attached to the substratum, and perhaps in the tail region. The ameba can, however, gradually increase its surface area in order to replace plasmalemma consumed in the formation of food cups or to allow for marked changes in form.

The block to cell division produced by deuterium oxide. Paul R. Gross, William Spindel and Delbert E. Philpott.

Renewed interest in the effects of D_2O on biological systems has stimulated recent studies on a variety of organisms. Although showing marked systemic toxicity in mammals, heavy water appears to have a more direct and severe effect upon rapidly proliferating cells and tissues. The present studies were undertaken in order to examine the D_2O effect on mitosis in systems where cell growth is not a source of experimental confusion. In *Arbacia* eggs, fertilization occurs normally in sea waters containing up to 25 volumes per cent D_2O. At higher concentrations, the number fertilized declines sharply, so that in 96% D_2O, only 2% of the eggs are fertilized whilst control cells in reconstituted normal water show 90–100% fertilization. The effect of D_2O on mitosis and cytokinesis is even more severe. In media containing more than 75 volumes per cent D_2O, mitosis is blocked at all stages; cells with cleavage furrows are arrested in the condition obtaining at the time of immersion. At 50% D_2O, many cells remain arrested; those not blocked suffer cleavage delays of three to four times the normal interval. Lower concentrations produce progressively smaller effects, and below 10% D_2O, the early cleavages proceed normally. The D_2O effect is at least partially reversible, even when the cells have been immersed in pure D_2O-sea water for more than an hour. Washing in sea water permits a significant number of cells to escape the block, and the resulting cleavages are usually irregular and multiple, so that if residence in D_2O has not been too prolonged, the treated eggs tend to "catch up" with the controls. Essentially identical findings are obtained with eggs of *Chaetopterus.* Electron microscope studies show the treated cells to have suffered multiple, localized gelations in the peripheral cytoplasm.

The effects of sperm on S^{35}-labelled Arbacia fertilizin. Ralph R. Hathaway.

The production of S^{35}-labelled *Arbacia* fertilizin has permitted observations of the fate of sulphur constituents of fertilizin during reactions with sperm. Previously reported experiments showed that, at times, the radioactivity was adsorbed to sperm, but that other conditions resulted in little or no association of S^{35} activity with sperm after mixing with labelled fertilizin. These results were interpreted as demonstrating a two-step reaction between sperm and fertilizin: (1) adsorption of fertilizin to sperm, (2) release from the sperm of part or all of the fertilizin molecule. These results have been confirmed and clarified in an experiment which permits observation of uptake and release of fertilizin-bound isotope in a single reaction mixture. Sperm and labelled fertilizin were mixed and centrifuged within 30 seconds. The supernatant (No. 1) was sampled for radioactivity. The sperm were re-suspended in the same supernatant and left at room temperature for 45 minutes, and then re-centrifuged. This supernatant (No. 2) was sampled. In 10 experiments it was found that supernatant No. 1 contained 28.0 ± 3.6% of the total radioactivity, while supernatant No. 2 contained 69.1 ± 10.0%. Therefore, in the early seconds of the reaction, the sperm adsorbed about 72% of the labelled material, and in 45 minutes these same sperm released all but about 31% of the radioactivity.

The fraction 31/72 represents a retention by the sperm of 42% of the radioactivity initially adsorbed. Correspondingly, 58% of the initially adsorbed material is released within ¾ hour.

Labelled material released by sperm was found in dialysis to be non-diffusible. Released substances are not appreciably adsorbed to fresh sperm when treated a second time. Similarly, reacted sperm were completely unable to adsorb fresh labelled fertilizin.

Heterologous sperm (*Spisula, Pecten, Chaetopterus*) adsorb fertilizin slower than *Arbacia* sperm, and do not release any labelled material. This may not be a surface reaction since these sperm are readily damaged by handling.

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Studies of fibers of acto- and paramyosin from lamellibranch muscle. TERU HAYASHI, RAJA ROSENBLUTH AND HAYES C. LAMONT.

Johnson and Szent-Györgyi have proposed that the "catch mechanism" of lamellibranch muscle is due to crystallization of the paramyosin component of these muscles by a pH shift within the muscle, the crystallization effectively "freezing" the muscle at any state of contraction. The mechanism was based on studies of glycerinated muscle and the pH dependence of the crystallization of paramyosin from solution.

This hypothesis was tested by making fibers from mixtures of acto- and paramyosin in which the paramyosin content varied from ca. 20% (Prep. A: *Venus mercenaria* whole extract) to ca. 5% (Prep. B: *Spisula solidissima* whole extract) to ca. 1% (Prep. C: *Spisula solidissima* actomyosin) and testing the fibers for changes in ATP-induced contractability and mechanical stiffness with pH. It was found that ATP-induced contractability for all preparations at the constant ionic strength employed showed a maximum at pH 7.0. On the acid side (pH 6.5–6.8) Prep. A showed the greatest decrease of activity, Prep. B a smaller decrease of activity, and Prep. C the least decrease. Mechanical stiffness was measured only for Preps. B and C, by the application of quick stretches and noting the change in tension. It was found that Prep. B showed a much greater increase in stiffness below pH 7.0 than did Prep. C. It is concluded that, while more experiments are needed, the results of the present investigation tend to support the crystallization hypothesis.

A study of membrane elevation in various marine eggs. L. V. HEILBRUNN AND THOMAS F. BYERS.

We have studied membrane elevation in the eggs of the sea urchin Arbacia, the clam Spisula, and the worms Chaetopterus and Hydroides. In the Arbacia egg, membrane elevation is a normal process and is accompanied by a breakdown of cortical granules. In the other eggs studied, membrane elevation does not occur normally, but is initiated by the same types of agents as those which cause membrane elevation in the Arbacia egg and is probably due to the same type of mechanism.

Cortical granules are not essential for the process. In the Chaetopterus egg, the cortical granules can be centrifuged to one end of the egg and then ultraviolet radiation causes membrane elevation in that part of the egg devoid of cortical granules. Sometimes, especially at the end of the breeding season, strong centrifugal force will shift the cortical granules in the Arbacia egg, and then after insemination, membranes will become elevated from regions devoid of these granules.

In the Arbacia egg immediately after membrane elevation the elevated membranes can be pushed back to where they came from by hypertonic solutions, especially those of $CaCl_2$ and $MgCl_2$.

If eggs are first exposed to isotonic sodium citrate solutions for a few minutes, no membrane elevation is possible (Arbacia, Chaetopterus).

Membrane elevation can scarcely be due to the swelling or osmotic pressure of a protein colloid, for it can be induced by 100% ethyl alcohol (Arbacia, Chaetopterus, Spisula). In aging Arbacia eggs, it can also be induced by dilute solutions of mercuric chloride.

Indirect evidence indicates clearly that agents which cause membrane elevation release calcium from the egg cortex (Chaetopterus).

We conclude that membrane elevation is due to a release of calcium from the cortex and that the calcium which is set free then exerts osmotic pressure against the vitelline membrane and pushes it out from the egg surface.

Supported by a grant from the National Science Foundation.

On the electron spin resonance of adenosine triphosphate. IRVIN ISENBERG AND ALBERT SZENT-GYÖRGYI.

Theoretical calculations by B. and A. Pullman show adenine to be a good electron donor since its uppermost filled energy level is high, indicating a low ionization potential. Work of this laboratory, currently in progress, indicates that the tri-phosphate group may be a good electron acceptor. Since adenosine triphosphate may fold so that the triphosphate group fits neatly over the adenine ring, the possibility exists that adenine may donate an electron to the triphosphate, forming a molecule in which charge is separated. Adenosine diphosphate does not permit such a folding.

Preliminary studies have been made on the possible spin resonance of ATP and ADP. Dry ATP powder *in vacuo* gave a low broad spin resonance signal. This signal was extremely complex and covered at least 600 gauss. This width is an order of magnitude above that which is ordinarily obtained for free radical signals. The half-width line broadening to be expected from two electrons separated 3.5 A is about 400 gauss. ADP did not present such a signal but gave only a low simple absorption line that was oxygen-sensitive and could have been produced by residual oxygen in the sample.

Aggregation and schooling in the marine snail, Nassarius obsoletus. CHARLES E. JENNER.

For the fourth consecutive summer a population of mud snails (*Nassarius obsoletus*) in Barnstable Harbor, Massachusetts, has been shown to undergo a change in distribution pattern . . . from dispersed, in which snails are present over extensive areas of the flat, to aggregated, with snails occurring in massive aggregations. This striking change occurs abruptly at the close of the egg laying season. In the aggregated state schooling behavior is observed in which snails move, often in large numbers, in the same direction. Motion pictures have been made showing this change in distribution pattern and also illustrating schooling behavior.

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Effect of temperature on electrical activity of nerve cord and cardiac ganglion of Limulus. PARASKEVI J. MAVRIDIS AND RICHARD C. SANBORN.

Electrical activity of the intermediate segments of the cardiac ganglion and of the region of the ventral nerve cord between the circumoesophageal ring and the first abdominal ganglion was recorded at various temperatures. At all temperatures, the activity of the ventral cord was less in posterior regions than in the region proximal to the ring.

As the temperature of the cardiac ganglion was lowered, the frequency of bursts of electrical activity decreased from 17/min. at 25° C. to 9/min. at 10° C. and 6/min at 6° C. Simultaneously the duration of each burst increased from 0.2 milliseconds at 25° to 0.6 milliseconds at 10° C. As the ganglion is warmed, the burst frequency increases until at 33° C. it is 23/min.

The behavior of the nerve cord is strikingly different. As the temperature is lowered from 25° C. the magnitude of the potentials recorded by external electrodes increases markedly. Thus, a typical cord displayed spikes with a maximum amplitude of 75 μV. at 25°, and spikes with amplitudes of 200 μV. at 10° C. As a cord is rewarmed, both the magnitude of the potentials and the frequency of bursts increase. There is a "hysteresis" effect. in that the frequency and magnitude of the bursts are higher in a cord which has been cooled than in one which has been maintained at a given temperature. Similar, though lesser, hysteresis occurs in the cardiac ganglion.

At temperatures higher than 33–35° C. both the cardiac ganglion and the ventral nerve cord display no activity. (Aided by a grant from the Purdue Research Foundation.)

Studies on the fertilization inhibiting action of Arbacia dermal secretion. CHARLES B. METZ.

Following certain conditions of stress *Arbacia* release a yellow-green fluid. This dermal secretion strongly inhibits fertilization (Oshima, 1921; Pequegnat, 1948) and fertilizin agglutination of sperm. The latter action involves inactivation of fertilizin combining sites (Metz, 1959). In view of this dual action it seemed of interest to investigate the mechanism of fertilization inhibition.

Dermal secretion was prepared by immersing *Arbacia* in tap water, rinsing them in sea water and collecting the resulting secretion. The pH of this dermal secretion was adjusted to 8.0. Inhibition of fertilization does not result from irreversible action on sperm for sperm washed from dermal secretion have undiminished fertilizing capacity. However, washing treated eggs seldom completely restores fertilizability although partial recovery with delayed cleavage is sometimes obtained.

Evidently the inhibiting action is upon the egg and involves the initial stages of fertilization. To localize the site of action of the dermal secretion, the egg surface was subjected to mild dissection and then tested for sensitivity to the dermal secretion. The mildest agent used was acid sea water. This removes the egg jelly. The fertilizability of such jellyless eggs, like control eggs, is reduced by dermal secretion. Evidently, then, the dermal secretion does not inhibit fertilization by action on the egg jelly. A further dissection of the egg surface is achieved by proteolytic enzyme treatment. Eggs treated with crystalline protease or trypsin followed by trypsin inhibitor (ovomucoid) and subsequently exposed to dermal secretion fertilize as readily as controls. They are insensitive to the fertilization inhibitor. Presumably the protease removes a site or substance with which the dermal secretion combines to block fertilization. This substance is evidently essential in normal fertilization. It probably is not fertilizin, for an excess of fertilizin fails to reduce the fertilization inhibiting action of dermal secretion.

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A light and electron microscope study of Limulus gill cartilage. PHILIP PERSON, DELBERT PHILPOTT AND ETHEL SUBEN.

In the light microscope, gill cartilage from young Limulus (1–2″ body diameter) is composed of small, closely packed cells (12–29 μ diameter) with deeply basophilic nuclei. A thin eosinophilic matrix surrounds all cells. With increase in body size, cell diameters and amount of matrix increase. In an 8″ animal cells of 250 μ diameter may be found. Young and old cells contain much water-soluble, alcohol-insoluble material. Cell contents and matrix are PAS-positive. In the very large cells referred to above, cytoplasm becomes filled with granules and small vesicles. Younger cells contain large vacuoles. With aqueous fixatives cells drop out of sections in large numbers. Alcoholic fixatives improve cell retention but distort the matrix. Electron microscope study of young cartilage reveals typical mitochondria, golgi apparatus, and endoplasmic reticulum. As matrix is formed, it is first a thin seam of amorphous material which separates adjoining cells. On either side of this amorphous material, chains of rod-like micelles are formed in a lamellar, appositional fashion. These rods seem to take origin as elements of the endoplasmic reticulum and other cytoplasmic components, which, as they approach the periphery of the cell, give the appearance of being incorporated into the matrix. The lamellae form concentric layers. There are also fibrous elements apparently lacking any periodicity in the matrix.

The effect of various metabolites on x-irradiated Tetrahymena pyriformis. JAY S. ROTH AND EILEEN FIORENTINO.

Tetrahymena pyriformis W grown in 2% proteose peptone, 0.2% yeast extract solution for three days were collected and washed three times with distilled water by gentle centrifugation. Aliquots of the cells were placed in small plastic boxes and irradiated with 300,000 r. During irradiation the cells were cooled with ice. Controls were treated similarly. Three types of experiments were run: I, the cells were irradiated in the presence of metabolite; II, cells were irradiated and metabolite added immediately afterward (1–3 minutes), and III, cells were irradiated and metabolite added 20–30 minutes later. Aliquots of control and irradiated cells were transferred to Warburg flasks containing buffer, and O_2 consumption measured at 10, 60 and 120 minutes after a suitable equilibration period. The metabolites used were sodium acetate, butyrate and pyruvate adjusted to pH 7.4; final concentration, 0.035 *M*. The stimulation of O_2 uptake of unirradiated cells by these metabolites was 99, 123 and 22%, respectively, for acetate, butyrate and pyruvate compared to controls without substrate. For I above, cells irradiated in water showed a depression of O_2 consumption of 13, 30 and 38% at 10, 60 and 120 minutes, respectively, after irradiation, while cells irradiated in the presence of pyruvate showed changes of + 80, + 17 and − 7% at 10, 60 and 120 minutes compared to unirradiated controls with pyruvate. Acetate was less effective in preserving respiration while butyrate had little action. For II above, pyruvate showed + 42, − 6 and − 27% changes in respiration compared to − 18, − 30 and − 42% for the water control. Acetate was effective, but less so than pyruvate, and butyrate had a slight protective action. In III, the protective action of pyruvate was considerably less than in II, although in some experiments it was almost the same. Cell appearance after irradiation correlated well with the protective action of the metabolites. (Supported by a grant from the U. S. Atomic Energy Commission.)

Cells of Limulus polyphemus in tissue culture. Richard C. Sanborn, Judith A. Haskell and Frank M. Fisher, Jr.

Cells which migrate from ovary, hepatopancreas, nerve, leg, and cardiac muscle have been cultured in hanging drops using a medium composed of inorganic salts, sugars, organic acids, and 5 to 10% sterile Limulus serum. Within a few hours after preparation, two types of cells appear at the margins of the explant of all of these tissues: (1) free, wandering, rounded cells in the body of the medium, and (2) spindle-shaped cells attached to the liquid-glass or liquid-air interfaces of the drop. Both of these cell types remain viable in culture for at least 30 days, but after 10 days increasing numbers develop granules and vacuoles. Free cells can be maintained in a healthy state for at least six weeks by the weekly replacement of the original explant with a fresh tissue mass.

These cells do not appear to be hemocytes (amoebocytes) which have also been maintained on clotted blood for as long as three months. When we use clotted blood as an explant under our cultural conditions, no free cells migrate into the medium, and the amoebocytes in the clot are smaller and more granular than the free cells which we have described. In cultures to which blood has been added in proportions greater than 10%, the blood contracts into a pseudo-tissue without cellular migration. Medium without blood has consistently stimulated earlier migration of cells from the explants.

Explants of peripheral nerve and muscle give rise to a preponderance of spindle-shaped cells while other tissues yield a higher proportion of round cells. Within a few days after preparation, acellular fibers appear at the margins of explants of hepatopancreas and ovary. As these grow, migrating cells appear to become entangled in this network. (Aided by grants from the National Science Foundation and the Purdue Research Foundation.)

The effect of urethan on the mitotic apparatus of the Chaetopterus egg. Herbert Schuel.

Cleavage of the eggs of the marine annelid, *Chaetopterus pergamentaceus*, is inhibited when they are placed in a 1% solution of urethan (ethyl carbamate) in sea water 5 minutes after fertilization. Relative cytoplasmic viscosity determinations made with the hand centrifuge show that the "mitotic gelation" does not take place.

Eggs put into 1% urethan at all stages of the first cleavage cycle are prevented from dividing. If the eggs are allowed to reach the stage where about half of them show cleavage furrows before they are exposed to urethan, the furrowing process appears to continue. Two minutes later all the eggs have two quite distinct blastomeres and a polar lobe. However, the furrows soon regress and the cells assume a spherical shape again, so that 15 minutes later only a small proportion of the eggs are seen to have completed furrows. These few eggs probably had already completed cytokinesis when they were exposed to urethan.

Cytological observations were made on preparations fixed in Bouin's fluid, sectioned and stained with Heidenhein's hematoxylin. Eggs put into 1% urethan 5 minutes after fertilization show no signs of the mitotic apparatus at a later time when in the sea water controls it is completely formed. If eggs are exposed to urethan at a time after the mitotic apparatus had already formed and are fixed 5 minutes later, no trace of the asters, centrioles or spindles can be seen.

This work was supported in part by a grant from the National Science Foundation to Dr. L. V. Heilbrunn.

Melanophore dispersing action of ataraxic drugs. George T. Scott.

During the winter, in association with Miss Martha Robinson, the observation was made that meprobamate (Equanil, Miltown) would cause dispersal of pigment in frog melanophores, an effect antagonized by adrenin. The drug was completely refractory in the hypophysectomized animal, in the isolated frog skin test and when placed in Ringer perfusate.

A comparative study using six drugs was undertaken on *Rana pipiens,* the sand-dab *Lophosetta maculata* and the skate *Raja erinaceae.* The M.E.D. on the frog for reserpine, chloropromazine (Thorazine), methoxypromazine (Tentone), and meprobamate was 1, 4, 6, 30 mgs. per 100 gms., respectively; azacyclonal (Frenquel) and oxanamide (Quiactin) were not effective. M.E.D. on *Lophosetta* for reserpine, chloropromazine and meprobamate was 0.7, 2, and 30 mgs.; azacyclonal, and oxanamide were likewise non-effective. M.E.D. on *Raja* for reserpine was 0.4 mg. Time required to cause dispersion of melanophore pigment was from 1 to 6 hours. Transfusion experiments revealed that 1 ml. of blood from a drug-darkened *Lophosetta* will cause darkening in a pale recipient which lasts for several hours. Two-tenths cc. of blood from a reserpine-darkened skate will cause expansion of frog melanophores. Melanophores of *Lophosetta* are unresponsive to injections of Pitressin or Pituitrin.

The experiments suggest strongly that in the frog and skate certain ataraxics cause secretion of intermedin. Since the melanophores of *Lophosetta* are apparently similar to those of the flounder *Pseudopleuronectes americanus* (Osborn, 1939) in being unresponsive to pituitary extracts, the source and nature of the active substance is uncertain. A neuro-humor from the dispersing nerve fibers is suspected although a melanocyte-stimulating hormone may nevertheless be involved. Further investigation is planned.

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Progenesis in digenetic trematodes and its possible significance in the development of present life-histories. Horace W. Stunkard.

Progenesis is the precocious development of sexual maturity in juvenile animals. The metacercariae of digenetic trematodes sometimes become mature in their intermediate hosts, usually crustaceans, aquatic insects or tadpoles. Both asexual generations and gravid metacercariae of a species of *Asymphylodora* occur in the fresh-water snail, *Amnicola limosa.* Eggs from these progenetic metacercariae were fed to laboratory-raised snails. Sporocysts and rediae were recovered from these snails, proving that a vertebrate host is not essential for completion of the life-cycle. There is a question whether progenesis is to be explained as an atavistic reversion or merely an abridgment of usual developmental cycles.

Metagenesis was first recognized by Steenstrup in coelenterates and parasitic flatworms but actually, alternation of generations was initiated and perfected in plants. The introduction of asexual reproduction was essentially similar in plants, sporozoans and digenetic trematodes. In all, it evolved in a parasitic generation. There is a close association between endoparasitism, asexual reproduction and metagenesis. The Digenea have a common ancestry with the Turbellaria. In certain rhabdocoels, asexual reproduction regularly alternates with sexual reproduction. Others, probably related to the ancestors of the Digenea, are parasitic in mollusks and echinoderms. If the ciliated larvae of ancient turbellarians, on invasion of a mollusk, were to form cyst-like structures in which the germinal cells separated and developed independently, a very efficient method of reproduction, polyembryony, would have arisen. This method is employed by the Digenea and progenesis may have completed the primitive life-history. With the advent of vertebrates, the former definitive hosts were eaten and the worms acquired new definitive hosts to which sexual maturity was deferred. As a result, the former definitive hosts were gradually reduced to intermediate, paratenic or transfer hosts.

On the significance of "voltage clamp" data in the "membrane oscillator." TORSTEN TEORELL.

The "membrane oscillator" is a model device aiming at a comprehensive understanding of the electrical behavior of excitable tissues. In its simplest form this model consists of: Compartment (1) containing N/10 NaCl (small volume)/ a negatively charged, porous membrane; compartment (2) containing N/100 NaCl. In the discussion which follows, a modification of this model was used which incorporated "leaky" pores as well. A steady D.C. current is applied with the cathode in (1) and the anode in (2). If the current density is increased in graded steps, the transmembrane potential undergoes oscillatory excursions, first damped, then above a certain current density ("threshold") undamped. The configurations of these oscillations are similar to those of the nerve action potentials. Arrangements in which the current is maintained constant are referred to as "current-clamp" experiments. Conversely, "voltage clamp" refers to an experiment in which the transmembrane potential is maintained constant and the current required to accomplish this is recorded. As the level of the maintained potential is varied in the model, the current undergoes a number of characteristic changes. In practically every respect these changes are similar to those observed by Cole, Hodgkin, Huxley, Katz, Tasaki and others on voltage clamp experiments on nerve tissue. (A representation of voltage clamp experiments as voltage current graphs also show a marked similarity with those obtained on physiological objects.) In all cases a short initial and terminal burst of current is observed, of opposite directions. The intermediate portion may show a "dip" below, or form a curve above the "resting level," or both. The current flow in the membrane oscillator reflects the changes in the internal membrane resistance. These changes are the resultants of the concurrent driving forces arising from electrochemical and hydrostatic pressure gradients. The initial and terminal current spikes, referred to as "capacity spikes" by many electrophysiologists, as well as the intermediate current curve are here due to resistance changes. In this respect, voltage clamp phenomena are simply interpreted by a straightforward application of Ohm's law. "Capacitive spikes" in the model are an expression of the time course of a resistance change and are accordingly only "apparent" (Teorell) or "anomalous capacities" (Cole). The characteristic "dip" often referred to as a "sodium inward current" has in the membrane oscillator no specific significance, it is merely one of many transitory states. The results suggest that alternative explanations of voltage clamp results are possible and that it may be advisable to search also for other hereto often neglected factors in the nerve mechanism such as water movements producing hydrostatic pressure changes across the excitable structures.

Nucleotide interactions associated with muscle actin transformation. KENNETH K. TSUBOI AND TERU HAYASHI.

Reactions in which the bound nucleotide of the muscle protein, actin, participate were examined using labelled ATP with the isotope P^{32} present in the two terminal phosphate groups. Polymerization (induced by salt) and further reversible depolymerization (induced by ATP in solution of low ionic strength) of actin solutions prepared from acetone dried muscle powder are associated with concomitant changes in the phosphorylated state of the bound nucleotide.

Polymerized actin (F-actin) and depolymerized actin (G-actin) contain bound ADP and ATP, respectively. Consecutive transformation of actin solutions from F- to G- and back to F- was found to occur without alteration in the amount of associated bound nucleotide. The mechanisms involved in the associated nucleotide changes were studied with the aid of labelled ATP. Examination of bound nucleotide on G-actin, following depolymerization of F-actin in the presence of externally added labelled ATP (APP*P*) and quantitative removal of excess, free, non-bound nucleotides on Dowex-1, revealed the presence of an ATP of identical labelling (equivalent distribution of isotope and S.A. of P groups) as that of the externally added ATP. Reversible depolymerization of F-actin in these experiments occurs, therefore, with a concomitant exchange of bound ADP with an ATP from the medium. Repolymerization of G-actin containing the labelled ATP (APP*P*) and isolation of the resultant F-actin by centrifugation, yields a product containing just one-half of the original isotope. The polymerization reaction is therefore accompanied by a direct dephosphorylation of the bound ATP and a release of the terminal P group into the medium.

Quantitative techniques for the removal of free nucleotides from actin solutions without removal of bound nucleotide were developed as a part of these studies. In spite of the inability of Dowex-1 to remove measurable amounts of bound nucleotide from G-actin solutions over a one-hour period, a complete exchange of bound ATP with externally added labelled ATP was found to occur within this period. This apparent paradox is being further examined.

A convenient and simple method for preparing nucleotide labelled actin preparations (either G- or F-) for further studies has also been incidentally provided from these studies.

Polyploidy in Mormoniella. P. W. WHITING.

Normally females are diploid, males haploid. Three mutations to male diploidy have been found. No reduction occurs in spermatogenesis, whether haploid or diploid, so that all sperm from diploid males are diploid. These males are as highly fertile as normal haploid. Their numerous daughters, which are triploid, lay many eggs most of which fail to hatch, presumably aneuploid. From the few (presumably euploid) eggs of unmated triploid females there develop haploid and diploid males. Triploid females mated to haploid males produce diploid and triploid females from their fertilized euploid eggs. The polyploid stock is maintained by alternating generations. For the "odd" generations diploid males are crossed to normal diploid females; for the "even" the triploid daughters are isolated and bred unmated. Triploid females mated to diploid males would be expected to produce from their fertilized haploid and diploid eggs, triploid and tetraploid daughters, respectively. Preliminary cytological study indicates this to be the case. The problem of genetic markers for separating these two classes of females has not yet been satisfactorily solved. Mutant eye colors are used as markers to separate diploid from haploid males. By using diploid sires whose sperm will have no effect to change eye color, separation of triploid daughters from tetraploid has been made, a few of the latter having been obtained. Markers used for separating the sires from their haploid brothers are not as yet satisfactory. It is proposed to remedy this condition by use of a second genetics locus. The problem of sex determination is still unsolved for Mormoniella. Females develop from fertilized eggs, males from unfertilized, seemingly without regard to ploidy.

Oxygen transport—a new function proposed for myoglobin. JONATHAN B. WITTENBERG.

A study of the secretion of oxygen into the swimbladder of fish, presented at this forum last year, gave strong evidence for the idea that the active transport of oxygen is mediated by an intracellular hemoglobin present in the cells of the gas gland. The present work explores the possibility that the penetration of oxygen into cells from regions of higher to regions of lower oxygen concentration might also be mediated by intracellular hemoglobins, *e.g.* myoglobin.

Lankester, in 1896, had noted the presence of intracellular hemoglobins in many tissues of marine invertebrates. Dissection of the animals available at Woods Hole revealed that the hemoglobin-containing tissues were frequently thick or bulky structures. Diffusion of oxygen into these structures would appear inadequate to supply their respiratory needs, suggesting that tissue hemoglobins serve to transport oxygen into the depths of these tissues.

Hemoglobin was chosen as a model compound. The penetration of oxygen through hemoglobin containing agar membranes was measured with the aid of the teflon-covered oxygen electrode. The penetration of oxygen through the same membranes in the absence of oxyhemoglobin was measured in the presence of carbon monoxide which converts all of the hemoglobin to the carboxy form.

The rate of penetration of oxygen through membranes containing oxyhemoglobin was in many cases about 1.6 times as great as through the same membrane containing carboxyhemoglobin.

On the basis of this finding a new function, oxygen transport, is proposed for myoglobin. The mechanism of this transport is not apparent and is the subject of continuing studies.

PAPERS READ BY TITLE

Changes in cytoplasmic structure following fertilization in Arbacia eggs. R. D. ALLEN AND L. BUNIM.

The echinochrome granules of sea urchin eggs respond to the presence of fertilized cortex (*i.e.*, passage of the cortical reaction) by migrating from various regions of the cytoplasm to a sub-cortical location within the first 10 minutes following fertilization (Allen and Rowe, 1958). A plausible working hypothesis to explain pigment migration would be contraction of fibrils connecting the pigment granules to the cortex after fertilization.

It appears not to be possible to decide on the basis of centrifugation data in the literature whether or not such fibrils could exist in the unfertilized egg, although it has been shown that these granules exhibit zig-zag translatory movements in the cytoplasm as if so moved (Parpart). At the height of the breeding season, centrifugal accelerations as low as 140 × g can sediment nearly all pigment granules and larger oil droplets without visibly sedimenting the lighter yolk. In some lots of eggs after the peak of the breeding season, many pigment granules remain suspended even after acceleration of 1800 × g has sedimented the yolk; application of 4000 × g displaces the pigment to the centrifugal pole, showing that it is still the heaviest particle present.

By applying various accelerations at different times between insemination and the completion of pigment migration, it was found that some pigment granules become either trapped in or attached to a subcortical gel layer as little as 30 seconds following insemination at 19–21° C. An increasing number of pigment granules remain in the cortex when eggs are centrifuged at 500–1900 × g during the first 10 minutes after insemination. It appears probable that at least some pigment granules can migrate to the cortical region in a centrifugal field of 500 × g, or a stress of about 3 dynes/cm^2. The evidence so far is compatible with our working hypothesis.

The effect of starfish toxin on amebocytes. F. B. BANG[1] AND A. B. CHAET.[2]

The occurrence of a heat-stable, dialyzable toxin in scalded starfish (*Asterias forbesi*) has been demonstrated previously. The present study concerns the effect of this toxin on coelomic fluid amebocytes of the starfish.

Through the aid of properly transmitted light, *in vivo* observations of amebocytes circulating through the dermal papillae were possible. Toxin injections (insufficient to induce autotomy) induced clumping of circulating amebocytes—a reaction reversed within 30 minutes.

Phase contrast studies showed that amebocytes, when removed from starfish and mixed on glass slides with equal volumes of concentrated toxin, round up and fail to send out the usual pseudopodial processes. Frequently, these cells not only lose their granules, but become glassy in appearance. In medium concentrations there seems to be no perceptible difference between the activity of the amebocytes in control and toxic fluids. However, if still lower concentrations of toxin were used (10–20%), the amebocytes became much flatter and thinner than usual, and more cellular clumping occurred than in the controls. This concentration is approximately the same as the final concentration of toxin present in the *in vivo* experiments noted above in which agglutination occurred. Amebocytes of the sand dollar (Echinarachnius), as well as the horseshoe crab (Limulus) were similarly affected by the starfish toxin.

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Amebocytes in coelomic fluid have been aseptically removed from starfish and kept alive in test tubes for at least two weeks (room temperature). Siliconization of glassware was not necessary, and the viability of cells was noted after two weeks by the pseudopodial processes sent out when placed on a glass slide, and by their ability to concentrate neutral red. Toxin (1–4%) had no demonstrable effect on the "cultured" amebocytes even after one week, but the cells were susceptible to 50% toxin. As in the *in vivo* experiments, these effects were reversible.

Experimental modification of the lunar rhythm of running activity of the fiddler crab, Uca pugnax. MIRIAM F. BENNETT AND FRANK A. BROWN, JR.

Groups of male fiddler crabs, *Uca pugnax*, collected from Chappaquoit were exposed to a light intensity of approximately 100 ft.c. from midnight through 6 A.M. and to one of less than 1 ft.c. from 6 A.M. until midnight for three consecutive 24-hour periods. Chromatophores of groups of 20 of these crabs were staged hourly from noon until they averaged 2.5 on the Hogben-Slome scale on the third day. These chromatophore readings were compared with those taken concurrently for control crabs which had been collected at the same times as the experimentals but were maintained in the laboratory at a constant light intensity of less than 1 ft.c. Comparisons indicated that the phases of the 24-hour cycle of color change for the light-treated animals had been shifted to times 5 to 6 hours (average for four different series—5.56 hours) earlier than comparable phases for the controls. Eight crabs of each group were then placed in chambers of activity recorders and their spontaneous running was recorded continuously for periods varying from 9 to 24 days under constant laboratory conditions. All groups showed activity cycles characterized by two maxima (12 to 13 hours apart) which moved to later times on succeeding solar days at the average lunar rate. Comparison of phases of the cycle of activity for the control and light-treated crabs indicated that phases of this lunar-day cycle of running had likewise been shifted to earlier times, but by 4 to 5 hours (average—4.4 hours). No tendency for the peaks of activity of the experimental crabs to move to the times of maximal activity for the controls was noted during the four different periods in which this experiment was performed.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Phasing of the rhythm of running activity of the fiddler crab. MIRIAM F. BENNETT AND FRANK A. BROWN, JR.

On July 28, 1959 and August 18, 1959, fiddler crabs, *Uca pugnax*, were collected from the beaches of Chappaquoit and Lagoon Pond. At Chappaquoit low tides occur ± 1 to 2 hours of lunar zenith and nadir, and at Lagoon Pond phases of the tidal cycle occur approximately four hours later than the comparable ones at Chappaquoit. The spontaneous activity of 8 crabs of each collection was recorded continuously under constant laboratory conditions for 15 (series 1) or 8 (series 2) days. It was seen that the phases of the lunar-day cycle of activity of the animals from Lagoon Pond occurred about five hours later than those for the Chappaquoit animals on day one, the first complete 24-hour period of observation immediately following collection. After this time, the peaks of maximal activity for the Lagoon Pond crabs gradually moved toward the times at which peaks of running for the Chappaquoit animals occurred, and by the seventh day of observation were synchronous with them. Maximal activity for crabs from Chappaquoit occurs under laboratory conditions near the times of lunar zenith and nadir, or within an hour or two of the times of low tides on their native beach. During the later days of observation (series 1), the phases of the lunar cycle for the crabs from the two different beaches remained in synchrony with each other, and continued to occur within one to two hours of the times of lunar zenith and nadir.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Actions of biogenic amines and derivatives on lobster neuromuscular transmission. F. BERGMANN, J. P. REUBEN AND H. GRUNDFEST.

Short-chain omega-amino acids and some derivatives activate the inhibitory synapses, while another derivative—carnitine—activates excitatory synapses. Other biogenic amines, *e.g.* epinephrine and norepinephrine, are inert even at 10^{-4} dilutions. Serotonin (5-HT) appears to enhance transmitter action, for, while the excitatory postsynaptic potentials (epsp's) are considerably increased, there is no significant change in resting potential or membrane conductance, such as might be caused by a synapse activator drug. Homologs produced by methylation of the terminal amino group, or the alpha-carbon, behave similarly. The phenolic hydroxyl may be largely responsible, since tryptamine and its monomethyl homolog, after first increasing the epsp then depress it and the ipsp. Other homologs of tryptamine, and gramine, have only depressant actions. In small part, however, the creatinine moiety present in all crystalline preparations of the 5-HT series may contribute to their action. However, creatinine itself is weakly active (10^{-3} w/v), while 5-HT is effective at 10^{-6} dilution. PEA (beta-phenethylamine) also depresses both epsp's, but tyramine, which has the phenolic hydroxyl, is inert. PEA and 5-HT are antagonists with respect to effects on epsp's. These actions are probably mediated by effects on transmitter activity, since the interplay of the drug effects is not accompanied by changes in electrical properties of the muscle fibers. However, it is not known whether the various drug effects described above are caused by enhancement or depression of release of the synaptic transmitter, or by some action on the transmitter substance itself.

Contribution of locomotion to the oxygen-consumption rhythm in Uca pugnax. WILLIAM J. BRETT, H. MARGUERITE WEBB AND FRANK A. BROWN, JR.

Crabs collected weekly at Chappoquoit were subjected in the laboratory to either normal light-dark changes or to reversed light-dark conditions. Both control animals and light-reversed animals were set up in respirometers and activity recorders maintained in constant low illumination. Averages for 12 to 14 crabs were used for all hourly values. The data were analyzed for possible solar and lunar cycles. The form of the average diurnal cycle for both locomotion and respiration for the 29-day period between July 21 to August 18 was just the reverse for the controls and the experimentals. In the controls the maximum for locomotion occurred at 2 A.M. and for respiration at 4 A.M. In the experimentals the maximum for locomotion occurred at 1 P.M. and for respiration at 3 P.M. Analysis of the daily records for oxygen-consumption and locomotion in controls and reversed crabs showed a lunar cycle with the two maxima generally 12 to 13 hours apart. The mean lunar cycle for the 29-day period revealed little if any difference between the controls and reversed animals in either oxygen-consumption or spontaneous activity. In oxygen-consumption one maximum occurred at one hour before lunar zenith in controls and experimentals, and the second maximum occurred at two hours before nadir in the controls and three hours before in the experimentals. For locomotion the maxima for controls and reversed animals occurred two hours before zenith and two hours before nadir. A coefficient of correlation of $+0.919 \pm .033$ $(N = 24)$ between the control animals and $+.545 \pm .146$ $(N = 24)$ between the reversed animals for the average diurnal cycle indicates that locomotion produces a considerable effect upon the form and magnitude of the oxygen-consumption cycle.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

A diurnal rhythm in response of the snail Ilyanassa to imposed magnetic fields. FRANK A. BROWN, JR., H. MARGUERITE WEBB, MIRIAM F. BENNETT AND FRANKLIN H. BARNWELL.

Ilyanassa were permitted to emerge from a narrow corridor into a symmetrically illuminated white field, under conditions of three kinds: without imposed magnetic field and with each of two kinds of imposed fields, one with lines of induction parallel to, and the other with lines at right angles to the initial path of the snails in the corridor. For each of 483 series of 60 snail-runs, two groups of ten in each field, obtained during the period July 6 through August 6, 1959, (a) the mean path of the snails in each magnetic field was expressed as difference from the control, and (b) the standard deviation of each was similarly expressed as difference from the control standard deviation. There was a daily rhythm in each of these differences. In the amount of turning, the snails turned to the right of the controls in the early morning hours, and to the left of the controls the rest of the day. The forms of the daily cycles for the two magnet orientations were very closely similar. In standard deviations, for both magnet orientations there were, parallelly, three sharply delimited periods of less deviation than for controls (5 A.M., 12 noon, and 7 P.M.) and more deviation than control for the other fourteen hours. For both of these effects of imposed magnetic field there was indication of an ability of the organism to differentiate between the two orientations of the imposed magnetic field, as shown by systematically differing relative influences of the two magnetic conditions as functions of time of day.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Effects of imposed magnetic fields in modifying snail orientation. Frank A. Brown, Jr., Miriam F. Bennett and William J. Brett.

An 18-cm. Alnico bar-magnet (equating at about 40 cm. the earth's field) was placed horizontally on a turn-table 14 cm. beneath an "orientation chamber" with the center of the magnet directly beneath the exit of a 3-cm. corridor from which snails were emerging. The amount of the deviation of the snails from a straight forward course was determined. During the period July 6 through August 6, 1959, 483 series of 60 snail paths were assayed between the hours of 5 A.M. and 9 P.M. Each series (60 snails) consisted of two groups of 10 snails without magnet present, two groups with magnet at right angles (south pole to right), and two groups with magnet parallel and south pole directed away from exit. The order in series was continually changed. For most of the period of study, the observer was uninformed as to the presence or absence, or orientation, of the magnet. Analysis of the results indicated decisively that the snails responded to the presence of the magnet. This was indicated in each of two ways, both with $p < .005$ using all data, and with a $p < .001$ when only data obtained between 7 A.M. and 6 P.M. were used. The first way was in the mean amount and direction of turning. The snails in the imposed magnetic fields turned more strongly to the left, on the average, than in the natural magnetic field. The second was in the dispersion of pathways expressed as standard deviations of the three groups of 20 in each series. Standard deviations averaged significantly greater in animals in imposed magnetic fields. Orientation of the experimental chamber in different compass directions did not alter the major features of turning which were characteristic of any given time.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Fluctuations in the orientation of the mud snail, Ilyanassa obsoleta, in constant conditions. Frank A. Brown, Jr., William J. Brett and H. Marguerite Webb.

Snails collected at intervals of 4 to 5 days from mud-flats at Chappaquoit were studied in white-enamelled boxes designed to measure the right and left turning of snails as they emerged from 3-cm. long corridors directed toward the magnetic south. Each box was illuminated by a large, diffusing light source located symmetrically above and slightly ahead of the corridor exit. Illumination from above was about 300 lux and from ahead and right and left, about 150 lux. Samples, each consisting of 10 snail exits, were taken between 5 A.M. and 9 P.M. during the period July 6 through August 6, 1959. The average angular deviation of the path from the straight line, measured for each of the 10 snails as they crossed a semi-circular arc with a radius of 3 cm. centered at the corridor exit, was determined. During the month of study there was a significant mean daily rhythm in the amount of turning with minimum at 5 A.M. and major maximum at 9 P.M. There was a secondary minimum at 6 P.M. There was also a highly significant daily rhythm of total dispersion of pathways with a minimum dispersion at 5 A.M., a gradual steady increase to 10 A.M., and then a steady decline to 9 P.M., the time of a second minimum in the daily period of observation. Finally, there was a gradual irregular increase in the mean daily amount of left-turning, during the 32-day period. The form of this fluctuation suggested a two-day lead correlation on mean daily barometric pressure. A coefficient of correlation of 0.4 ($P < .05$, $N = 30$) was found between these.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Neural involvement in the firefly pseudoflash. Albert D. Carlson.

When a firefly is made hypoxic it develops an "hypoxic glow," and if it is then returned to air a bright "pseudoflash" usually occurs. According to the tracheal end-cell valve theory the pseudoflash is due to entry of oxygen through temporarily inactivated valves (*i.e.*, is independent of neural control). However, neural involvement in the pseudoflash is indicated by the following facts: (1) Under standard conditions a pseudoflash of high intensity can be induced in 5 seconds in a firefly which has been actively flashing spontaneously, but requires 30 seconds in a quiescent individual. (2) When pseudoflashes are induced repeatedly in a firefly the intensity of the successive pseudoflashes decreases progressively and the time required to reach maximum hypoxic glow intensity increases. However, if the specimen is stimulated electrically or mechanically while in air between bouts of hypoxia, the intensity of successive pseudoflashes and rate of rise of the hypoxic glow can be maintained. (3) Low frequency stimulation during early hypoxia increases the hypoxic glow intensity. (4) Pseudoflashes can be more rapidly induced in a decapitated animal by injection of 10^{-3} M serine than in an uninjected animal. (5) Transection of the nerve cord between the fifth and sixth abdominal ganglia greatly reduces the pseudoflash in the regions of the lantern innervated from the sixth, but leaves unaffected those regions innervated from the fifth.

Peripheral aspects of firefly excitation. James F. Case and John Buck.

Even with electrodes directly in photogenic tissue, the response latency of the adult *Photuris* is 65 milliseconds at 25°. This long delay is especially curious in that the actual light emission, involving coordination of several hundred thousand photocytes, may occupy only 50 msec. The possibility of at least two steps in the activation of the lantern is suggested by the fact that an intense stimulus (for example, 5–10 msec., 60–150 volts) may induce a short-latency flash (15 msec.) in addition to the usual long-latency flash, the minimal stimulus for which is 1 msec., 3 volts. This "quick flash" shows the same sorts of facilitation and strength-duration relations as the normal (slow) flash, is of comparable intensity and form, and appears to involve the same tissue. However, the quick flash differs sharply from the slow flash in its temperature response, showing scarcely any change in latency, and it can "follow" a much higher frequency of stimulation. The quick flash might represent either direct stimulation of photocytes or the by-passing of some intermediate echelon in the activation sequence. However, the quick flash latency probably represents more than the actual chemical delay of photogeny, for the quick flash of the larva is essentially instantaneous. Since tracheal end-cells are absent in the larva it is tempting to guess that the first step in the activation of the adult lantern—the step by-passed by intense stimulation—is the nervous stimulation of end-cells which then coordinate the flashing of the photocytes in an as yet unknown fashion. With regard to differential responses at the effector level, we find that various agents such as hypoxia and eserine can dissociate the over-all flash into asynchronous sparkling of tracheal "cylinders" or even of individual photocytes.

Physiologic activity of nerve extracts. A. B. Chaet and R. A. McConnaughy.

While working on unrelated problems, it was discovered that hot water extracts of starfish nerves (*Asterias forbesi*) induced the shedding of gametes when injected into small starfish (4–5 inch). After 15 radial nerves were isolated and washed, they were suspended in a test tube containing 15 cc. of sea water. This supernatant was then heated (1½ minutes at 76° C.) and considered a control. The 15 nerves were re-suspended in sea water and heated (1½ minutes at 76° C.). This supernatant, the first extract, when injected into recipient starfish induced shedding in 36–39% of the animals. Each starfish received an extract obtained from approximately 5 radial nerves (0.15 cc./gm. starfish). It is significant to note that upon autopsy, only 10% of the gonads appeared to be "mature," whereas 52% were "very poor." This procedure did not extract all of the shedding substance, since a second extraction using the same nerves induced shedding in 38–41% of the animals. Various attempts at more efficient extractions have been unsuccessful.

The shedding reaction was not due to the presence of potassium since the shedding substance was heat-labile and non-dialyzable. Furthermore, direct potassium analysis has shown that some extracts contained no more potassium than controls and still they induced shedding.

Recipient starfish failed to respond to the shedding substance after the middle of July, due to the lack of gametes (almost all gonads were "very poor"). However, the possibility that the concentration of the shedding substance available in the nerve fluctuates seasonally is being tested.

When starfish were injected with the above extracts (either fractions 1 or 2), it was noted that 2.3–3.5 times more fluid was excreted by the experimental animals than the control. This diuretic action was not decreased when the extracts were autoclaved but was slightly lowered by dialysis.

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Relation of x-ray dosage to modifications in fertilization and early development of Arbacia punctulata. RALPH HOLT CHENEY AND CARL CASKEY SPEIDEL.

Systematic studies of effects of a graded series of x-ray irradiations were made, in each instance, upon gametes from a single pair of sea urchins. Dosages ranged from 0.5 to 600 kiloroentgens, principally 1, 2, 4, 7.5, 30 and 60 kr. Experimental material was exposed in "Position A" to cross-firing from Coolidge x-ray tubes yielding 4940 r per minute. Observations by each of us were made synchronously of mass cultures and, by special techniques, aliquots were examined momentarily with the compound microscope. Events were followed continuously for the first few hours, and later at frequent intervals during critical periods of differentiation of blastulae, gastrulae, and plutei. Irradiation effects were observable not only on the cells themselves but also on the surrounding jelly, as evidenced by adhesive clumping of the eggs accompanying higher dosages. Direct relationship existed between dosage and degree of retardation in streak formation, prophase amphiaster, first and subsequent cleavages, blastula, and later development.

Our method distinguished clearly between the higher dosages employed in each series. Appreciable deviation from normal development was detectable also after 0.5 kr. This indicated the sensitivity of *Arbacia* to irradiation. Extent of retardation and percentages of abnormal types resulting from changes initiated by x-ray irradiation were similar in many respects to those found by us in parallel experiments with 2537 Å ultraviolet irradiation. Such similarities of equivalent abnormalities gave an indication of comparable dosages of these two physical agents.

Illustrative ciné-photomicrographs were obtained. Pictures taken at slow speeds vividly revealed changes during early development; those at normal speed showed individual deviations in movement and structure during later development.

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A rapid method for determining respiratory quotients in small animals. C. LLOYD CLAFF, F. N. SUDAK, J. R. BLUMSTEIN AND F. J. TEYAN.

A method for the rapid determination of respiratory quotients of small animals is described. R.Q. measurements can be obtained every 20–25 minutes. The apparatus used consisted of a Pauling paramagnetic oxygen analyzer connected either in series or in parallel with a carbon dioxide analyzer (titrimetric). Using an open ended system, dry, CO_2-free air was drawn past the animal, confined in a brass metabolism chamber, at the rate of 325 cc./min. A sample (100 cc./min.) of the effluent air was bypassed through the O_2 analyzer and directly analyzed for the partial pressure of oxygen. The volume of O_2 was calculated from the difference in the partial pressure of the O_2 in the affluent air (room air) and the partial pressure of the O_2 in the effluent air, times the air flow rate divided by the barometric pressure at the time of measurement. The latency of the system was 3–4 minutes. Partial pressure of O_2 was read every thirty seconds for a 5–6 minute period and the average partial pressure for this time period was used in the calculation of oxygen consumption.

The volume of carbon dioxide added to the effluent air by the animal was measured titrimetrically three minutes later, using a known concentration of barium hydroxide, and thymolpthalin as the indicator. The time required for a carbon dioxide analysis was 6–8 minutes, depending on the size of the animal. Alternate O_2 and CO_2 determinations were made in duplicate. The results of a typical experiment in the determination of R.Q's of a fasting and non-fasting 163-gram rat were: non-fasting R.Q, 0.970, 0.970; 48-hour fast 0.702, 0.726; non-fasting R.Q. 24 hours later, 0.964, 0.949.

Metabolism-body temperature relationships in cold acclimatized rats treated with 2,4 Dichlorophenoxyacetic acid ("2,4-D"). C. LLOYD CLAFF, F. N. SUDAK, F. J. TEYAN AND J. R. BLUMSTEIN.

The effect of 2,4 Dichlorophenoxyacetic acid on chemical thermoregulation was studied using fasted rats acclimated to 2.0° C. (3-week acclimatized period). Unacclimatized fasted rats served as controls. The "basal" metabolic rate, as measured by carbon dioxide production, was greater (150%) in cold acclimatized animals. "2,4-D" had no effect on the increase in "basal" metabolic rate which occurs during cold acclimatization. Evidence is presented which indicates that "2,4-D" does not interfere with chemical thermoregulation when cold acclimatized rats are exposed to low ambient temperatures.

The irreversible effects of caffeine on striated muscle. D. M. CONWAY AND T. SAKAI.

Caffeine can activate striated muscle without significant change in membrane potential, or even after the resting potential has been completely abolished by isotonic K_2SO_4, as found by Axellson and Thesleff with the frog sartorius, and confirmed by us using this muscle, and also the retractor penis muscle of the turtle *Chrysemis picta.* It had earlier been observed in this laboratory that frog muscle completely depolarized by 120 mM/l. KCl goes into irreversible contracture, just as normal muscle does on quick freezing and subsequent thawing. We suspected, therefore, that the effect of caffeine may also be irreversible, which would seem to limit somewhat the significance of the above observations. Recovery in normal Ringer after treatment with isotonic K_2SO_4 alone is complete, as judged by the isometric tetanus tension. There is a significant loss of tension, however, after caffeine treatment. The loss of tension is proportional to the concentration of the caffeine and the duration of the treatment. There is 95% recovery of the resting membrane potential (measured with intracellular microelectrodes) after 20 minutes' treatment with normal Ringer following 95 mM K_2SO_4 contracture. However, after caffeine contracture there is great scatter of values after 30 minutes' recovery, ranging from 10% to 75% of the normal. The threshold concentration for contracture at room temperature is 0.5×10^{-3} w/v, but lowering the temperature decreases the threshold. No contracture is elicited by 0.25×10^{-3} w/v at room temperature unless the solution is cooled to 0–4° C. The membrane potential is only slightly lowered on caffeine treatment and lowering the temperature causes a further drop in potential, but the value still remains above the critical potential.

The effects of caffeine on single fibers of the frog sartorius were studied with the phase-contrast microscope. Isotonic K_2SO_4 was found to have no effect on the fibers, but a concentration of 10^{-4} w/v caffeine induces a succession of contraction waves, followed by spontaneous twitching. A third stage involves disintegration of fiber structure. These observations were substantiated by electron microscopy.

Procaine has no effect on the caffeine contracture, neither has curare.

The sensitivity of the non-propagating muscle to longitudinal current. D. M. CONWAY AND T. SAKAI.

Our laboratory provided evidence that non-propagating striated muscle can be triggered by longitudinal electric fields of about 4 V/cm. We suspected that the field requirement of these muscles was high because of their low sensitivity to current. We studied, therefore, the question of whether under appropriate experimental conditions this sensitivity can be increased. Using the retractor penis muscle of the turtle we found that when it was recovering in normal Ringer from K depolarization, while the propagation had not yet returned, an a.c. or d.c. stimulus of about 2 V/cm. will bring about a substantial shortening along the length of the muscle, as recorded by the "fluorescent marking" method of Csapo and Mashima. If the recovery of propagation is delayed by using low temperature, or by having the depolarizing solution Ca-free, the sensitivity of the non-propagating muscle to current is prolonged. The recovery solution need not be normal Ringer; it is sufficient to lower the [K], *e.g.*, from 16 to 12 mM/l. In this case stimulation with 2 V/cm. longitudinal a.c. between two platinum plate electrodes in a square chamber produces 75% of the maximum shortening along the length of the muscle, when it is still non-propagating, as tested by stimulating one end only.

It was found that if the muscle is treated with 10 mM K_2SO_4 solution it becomes non-propagating within 5 minutes, and a longitudinal field of 2 V/cm. a.c. results in about 60% of maximum shortening.

Also, if 10% methyl alcohol is put on the muscle for about 5–10 minutes, propagation is lost, but a stimulus of only 1.5 V/cm. produces almost 50% of the initial shortening. In this condition the membrane potential is reduced to 55 mV. Recovery of normal excitability and tension was complete in a few minutes on return to normal Ringer.

Latency period, contraction time and fiber diameter. D. M. CONWAY AND T. SAKAI.

A correlation has been suggested (Peachey, *Science,* 1959) between the rapidity with which the active state develops, the diameter of the myofibril, and the density of the endoplasmic reticulum surrounding it. We have examined the duration of mechanical latency and the time required for the development of maximum tension, and attempted to correlate them with fiber diameter and density of endoplasmic reticulum in a typically fast and a typically slow striated muscle, *i.e.,* the frog sartorius and the retractor penis of the turtle.

Both muscles were stimulated at 27° C. with a.c. 60 c/s, 1 V/cm. for 1 second. Two recording channels of an electroencephalograph were used, one leading from the transducer and recording the tension, the other registering the duration of the stimulus.

The latency period in the sartorius is about 15 msecs. and the maximum tension measured isometrically is reached at 100 msecs. The latency period for the retractor penis is about 60 msecs. and the maximum tension is reached only after the termination of a one-second stimulus.

Electron micrographs were made of the two muscles and it appears that the myofibrils in the retractor penis are of the same diameter or thinner and the endoplasmic reticulum is more dense than that of the sartorius.

The diameter of the uterine muscle cell is only about 10 times greater than that of the myofibrils of the frog sartorius, but the latency period of the uterus is more than 100 times longer than that of the sartorius. The time required for development of maximum tension is about 60 times longer.

A general theory of membrane elevation in marine eggs. DONALD P. COSTELLO.

During past years, there have been so many theories proposed to explain membrane elevation in echinoderm eggs that it is almost presumptuous to contribute more on this subject. However, as was long ago pointed out by Lillie, a comparative study of the cortical response to activation of eggs of animals of several phyla may lead to a more generalized view than is possible by studying only echinoderms. Thus far, we have studied various aspects of the cortical response to fertilization of Arbacia, Asterias, Echinarachnius, Dendraster, Nereis, Hydroides, Spisula, Diopatra, Chaetopterus, Urechis, etc., using such methods as alkaline

NaCl treatment and/or centrifuging. In Arbacia and related forms, the vitelline membrane elevates (Heilbrunn) while a colloidal material accumulates (Loeb) in the perivitelline space. The membrane subsequently hardens and thickens into the fertilization membrane. In Nereis, the jelly precursor granules of the "cortex" release material which accumulates as a jelly external to the vitelline membrane (Lillie, Just, etc.). Other forms vary differently.

Our general hypothesis assumes, as do many earlier workers, that the basic similarity involved is the release of a "colloidal" substance by the egg on activation. The differences between the various eggs (magnitude of, timing, or absence of membrane elevation) might be accounted for in terms of (1) differences of porosity (or permeability to the colloid) of the vitelline and/or other membranes, (2) their nature, thickness, elastic tension, toughness or strength, (3) the time of release in relation to time of fertilization, (4) amount of substance released initially or over a longer period, (5) whether release is from the entire egg cortex, from localized areas, or from local points varying with time, (6) the molecular or micellar size of the released colloid.

For example, no membrane elevation need occur in an egg with a very tough, non-elastic, non-porous membrane, whose tension might greatly exceed the order of magnitude of the colloidal osmotic forces involved.

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A fundamental difference in the vitelline membranes of the eggs of marine annelids. D. P. Costello and Catherine Henley.

Alkaline sodium chloride at a pH of 10.5, isosmotic with sea water, has been known for some years to be an effective agent for freeing certain eggs of their vitelline membranes. However, the eggs of the annelids studied thus far fall into two very distinct groups as regards vitelline membrane behavior in this medium. Fertilized eggs of Nereis and Diopatra, after treatment, show an elevation of the membrane. Then, as the solution is changed a few times (to eliminate the divalent ions of sea water), the widely distended membrane ruptures at one point, the egg rolls out, and the empty membrane, consisting of two separate layers which are usually clearly discernible, remains. These eggs do not form sticky aggregates during the membrane-removal process.

In the eggs of Hydroides (either fertilized or unfertilized), on the other hand, after two or three successive washings with alkaline NaCl, the vitelline membranes become sticky, the eggs clump together, the membranes elevate and then *dissolve* to allow the denuded eggs to drop from the aggregate.

Novikoff (1937), using an alkaline NaCl treatment for the eggs of Sabellaria, found that the eggs adhered to each other to form aggregates. Then, as the membranes dissolved, the aggregates broke up and released the eggs, with adherent jelly.

There is obviously a fundamental difference in the vitelline membranes if, in one case, they become sticky and dissolve in the alkaline NaCl, and, in the other, are distended by the colloidal material swelling under them but remain relatively intact with the same treatment. In both cases, some colloidal material swelling in the perivitelline space accounts for the membrane elevation occurring during treatment.

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Electrophoretic analysis of the distal retinal pigment dark-adapting and light-adapting hormones in the prawn Palaemonetes. Milton Fingerman, William C. Mobberly, Jr. and Charles W. Philpott.

The object of this investigation was to determine the electrophoretic characteristics of the hormones controlling migration of the distal retinal pigment in *Palaemonetes.* Forty eyestalks were extracted in 0.2 ml. distilled water and centrifuged. The supernatant was applied with the aid of a hot-air blower to a one-half inch wide Whatman No. 1 filter paper strip. In some experiments 0.1 M sodium hydroxide-boric acid buffer of pH 9.0 was used and in other experiments 0.1 M sodium phosphate–0.05 M citric acid buffer of pH 5.2. The voltage used was 500 volts and the current 0.1–0.2 milliampere. Electrophoresis proceeded for two hours. The portion of the strip where the extract had been applied and the first three inches on the anodal and cathodal sides of the origin were washed for 30 minutes in 0.3 ml. sea water. The material washed from the three parts of the strip was then assayed on one-eyed *Palaemonetes* kept in black enameled pans under an illumination of 33 ft. c. At pH 5.2 the dark-adapting and light-adapting hormones were electropositive. When both substances were present in the same extract, the light-adapting effect preceded the dark-adapting one. At pH 9.0 the light-adapting hormone was again electropositive. However, about one-third of the dark-adapting material had migrated toward the anode. Presumably, the charge on some of the molecules of dark-adapting hormone had been reversed. The isoelectric point of this substance is presumably near pH 9.0. In other experiments both the dark-adapting and light-adapting hormones were found to be inactivated by trypsin.

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Influence of long exposure to light and darkness upon the distal retinal pigment light-adapting hormone of the prawn Palaemonetes. MILTON FINGERMAN, CHARLES W. PHILPOTT, MURIEL I. SANDEEN AND WILLIAM C. MOBBERLY, JR.

One group of *Palaemonetes* was placed in darkness and another group in white enameled pans under an illumination of 33 ft. c. for 14 days. After this period of adaptation, the supraesophageal ganglia were dissected from specimens in both groups and extracted in sea water. The concentration was 10 organs per ml. The extracts were assayed for distal retinal pigment light-adapting hormone on one-eyed *Palaemonetes* in black enameled pans under an illumination of 33 ft. c. The supraesophageal ganglia of the *Palaemonetes* that had been in darkness contained a greater amount of light-adapting hormone than the supraesophageal ganglia of the specimens that had been in light. No significant difference, however, was found between the quantities of light-adapting hormone in the supraesophageal ganglia of freshly collected specimens and of the prawns that had been illuminated for 14 days. By use of a dosage-response curve it was found that the supraesophageal ganglia of the specimens that had been in darkness for two weeks contained at least four times as much light-adapting hormone as the supraesophageal ganglia of freshly collected specimens and of the specimens that had been illuminated for 14 days. This difference in quantity of light-adapting hormone is evidence for the fact that this substance in the supraesophageal ganglia is normally involved in regulation of distal retinal pigment migration.

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Hormonal regulation of the distal retinal pigment of the shrimp Crangon septemspinosus. MILTON FINGERMAN, MURIEL I. SANDEEN, WILLIAM C. MOBBERLY, JR. AND CHARLES W. PHILPOTT.

Extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, from *Crangon* were assayed on *Crangon* kept in black enameled pans under an illumination of 31 ft. c. Within this intensity range the distal retinal pigment was in a position intermediate between the fully light-adapted and dark-adapted ones. A light-adapting effect alone was apparent. However, when eyestalk extracts of *Crangon* were assayed on *Palaemonetes* a dark-adapting effect followed the usual light-adapting one. When eyestalks of *Palaemonetes* were assayed on *Palaemonetes* light-adapting and dark-adapting effects were found but when extracts of eyestalks of *Palaemonetes* were injected into *Crangon,* light-adaptation alone was observed. When eyestalks of *Crangon* were subjected to filter paper electrophoresis at pH 9.0 the light-adapting hormone was found to be electropositive and the dark-adapting substance electronegative. The quantities of both hormones recovered were small. No light-adapting hormone had migrated toward the anode. Therefore, the small amount of dark-adapting substance that had migrated toward the anode was not masked by a light-adapting one as must have been the case when freshly prepared eyestalk extracts of *Crangon* were injected into *Crangon.* The distal retinal pigment of specimens of *Crangon* kept in black containers under an illumination of 31 or 33 ft. c. showed a 24-hour cycle of migration. This pigment more closely approximated the fully light-adapted state at this light intensity range about 7–8 P.M. than at any other time of day and was least light-adapted about 4 A.M.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

A reliable collecting locality for Pelomyxa palustris. J. L. GRIFFIN AND R. D. ALLEN.

Pelomyxa palustris (Greef) (the type species for the genus *Pelomyxa*) is important not only taxonomically, but also as material for many kinds of cell research. This species of ameba may regularly be found in a shallow inlet, near Dillingham Avenue, of the large fresh-water pond which lies between Lawrence High School and the center of the town of Falmouth, Mass. Mast and others have collected *P. palustris* at this locality since 1934. Many other ponds in the Falmouth region have yielded *A. proteus* and smaller species, but not *Pelomyxa*.

It would be desirable if more investigators were to study *P. palustris* and arrive at their own conclusions regarding the best taxonomic designation for the giant ameba, variously called *Chaos chaos, Chaos carolinensis, Amoeba carolinensis,* and *Pelomyxa carolinensis.* It seems to us superficial and misleading to classify the giant ameba as a *Pelomyxa* on the grounds that it is multinucleate. *P. palustris* and the giant ameba differ from one another so widely in form, manner of locomotion, nuclear morphology, cytoplasmic inclusions, preferred food, and enzyme content that it seems best to maintain the giant ameba in a separate genus, *Chaos,* until the application of modern methods has provided sufficient evidence with which to revise the currently rather confused taxonomy of the large, free-living amebae.

Glucose-6-phosphatase in sea urchin eggs. PAUL R. GROSS AND GILLES COUSINEAU.

Ca-Mg-free *Arbacia* eggs were homogenized in cold KCl containing 0.001 M EDTA. With this preparation as an enzyme source, it was possible to obtain zero-order liberation of inorganic phosphate from a reaction mixture containing histidine buffer at pH 6.5 and the sodium salt of glucose-6-phosphate. The homogenates were then fractionated by differential centrifugation, a procedure which yielded pigment, yolk, mitochondrial, and microsomal pellets. The final supernatant, remaining after sedimentation of the microsomes at 80,000 × g for 30 minutes, was retained as the "soluble phase." The pigment fraction was not used for enzyme assay, since the echinochrome interferes with the phosphate determination. The microsome fraction is a collection of small RNP particles and a few very small vesicles; it accounts for 6.8% of the cell's TCA-insoluble N. The mitochondrial fraction is composed primarily of mitochondria with a few contaminating microsomal particles; it accounts for 5% of the TCA-insoluble N. The yolk pellet, which has more than 30% of the TCA-insoluble N, contains yolk particles and a few mitochondria. The soluble phase contributes 9% of the cell's TCA-insoluble N. When these fractions were assayed for glucose-6-phosphatase activity, the following specific activities were measured (μg P/mg. N/25 min. incubation): whole homogenate, 18.6, soluble phase, 0, microsomes, 2.0, mitochondria, 16.0, and yolk, 30.7. The data indicate that the enzyme is localized to a considerable extent in the yolk particles themselves or in some contaminant of that fraction (other than mitochondria). They are in sharp contrast to the situation in mammalian liver, where the enzyme is almost exclusively microsomal. We relate this finding to the absence of a highly-developed membranous component of the endoplasmic reticulum in the *Arbacia* egg.

Ultrastructural changes in regenerating rat liver. PAUL R. GROSS, MURIEL FEIGELSON AND DELBERT E. PHILPOTT.

Cells of normal and 46-hour regenerating liver from male Wistar (Carworth Farms) rats appear to differ most clearly in the following ways: *Endoplasmic reticulum* (E.R.): Present in normal liver, confined to "patches" in the cytoplasm. Found frequently in parallel associations of cisternae. The entire complex (including RNP particles) is more electron-dense than the cytoplasmic matrix. In regenerating liver, parallel cisternal systems are rarely found; when found, the membranes undulate. E.R. membranes here are often closely associated with the outer membranes of mitochondria. The entire complex is *not* more electron-dense than the cytoplasmic matrix. *RNP particles:* In normal liver, these are most obviously associated with E.R. membranes, although some are free; many particles of similar size in cytoplasmic matrix are less electron-dense. In regenerating liver, there is a marked increase in the total number of *free* RNP particles; these, profusely scattered in the cytoplasmic matrix, are quite as dense as those associated with the E.R. membranes. *Ground cytoplasm:* In normal liver, there are large "hyaline" areas of low density; in regenerating liver, the ground cytoplasm is as dense (because of the large number of RNP particles) as are the nucleoplasm and the mitochondrial matrices. *Free fat droplets:* These are almost never found in the cytoplasm of normal liver cells, but are quite commonly observed in the regenerating material. They are internally structureless and more dense (osmiophilic) than other inclusion bodies. *Nuclei:* These are spherical or ellipsoidal in normal liver, with one or two nucleoli of granular (150–350 Å), but otherwise irregular structure. In the regenerating liver, many nucleoli are encountered with puckered surfaces, surrounded by cytoplasmic matrix free of large particles. Dense, periodic structures are found within such nuclei, suggesting condensed or condensing chromosomes rather than nucleoli.

Functional complementarity of leucine mutants of Neurospora. S. R. GROSS.

An analysis of sixty independent mutational events at the *leu*-3 locus of *Neurospora* indicates a complex intralocus functional heterogeneity. Although each mutation presumably leads to the loss of the same enzymatic function, the mutants fall into four categories with regard to the restoration of enzymatic activity in heterocaryons. Members of group A (31 mutants) do not demonstrate restoration of activity with members of groups B, C or D. Group B (13 mutants), on the other hand, in combination with members of group C (14 mutants) demonstrate a restored activity. Group D mutants (2) restore activity in combination with some members of groups B and C. It seems certain that specific reorganization of enzyme protein occurs in the cytoplasm of heterocaryons. A study of the spatial orientation of the four functional classes within the *leu*-3 locus as well as the specific alterations in protein structure is in progress.

Membrane resistance and rectification in muscle stimulated by rapid cooling. RITA GUTTMAN AND LEONARD ZABLOW.

After rapid cooling from about 25° C. to 5° C., no significant change in membrane rectification or capacitance of *Mytilus* smooth muscle could be detected. However, preliminary experiments indicate a percentage increase in membrane resistance equal to or greater in magnitude than that which occurs when sea water is cooled through this temperature range. The measurements were made by displaying as a Lissajous figure the current through and the voltage across the muscle, one end of which was treated with sea water containing 20 times the usual amount of KCl. The current was of triangular wave-form, with a frequency of 5 cycles/second. The resistive component of the impedance was balanced out by a bridge circuit, leaving a distorted ellipse on the screen, whose shape depended on the capacitance and rectification, changes in which could thus be determined. Since it was not possible to complete these measurements until some seconds after the onset of contraction (rapid cooling is an effective stimulus for this muscle), this change may well be unconnected with activity. Our method did not permit observation of transient changes of resistance less than 0.5 second.

Since previous investigations by one of us (R. G.) indicated that stimulation of *Mytilus* smooth muscle by rapid cooling is accompanied by changes in demarcation potential, we hope to investigate the possibility of earlier and smaller transient changes in future work.

Del Castillo and Machne could detect no change in membrane conductance of single frog sartorius fibers (which cannot be stimulated by cooling) greater than that which occurs on cooling Ringer's solution. Elucidation of whether rapid cooling affects electrical parameters of *Mytilus* muscle, where cooling *is* an effective stimulus, may indicate whether the cooling stimulus affects the contractile mechanism directly or triggers it through membrane changes.

The effects of podophyllin on the fertilized eggs of Chaetopterus. CATHERINE HENLEY AND D. P. COSTELLO.

Fertilized Chaetopterus eggs were treated with various concentrations of podophyllin (the stock solutions of which were made up in distilled water), beginning 5 minutes after insemination, for periods of 42 to 60 minutes. Cytological preparations were made of samples of all treated and control eggs.

With concentrations of 0.0055 to 0.1 mg./cc., a characteristic series of changes was observed. For the initial 90 seconds, the first maturation metaphase was unaffected. About two minutes after the beginning of treatment, however, there were many eggs in which the asters had completely disappeared, and by 5 minutes after the beginning of treatment, all asters and spindles had disappeared. About one minute later, the egg chromosomes were surrounded by a nuclear membrane; in some instances, the chromosomes remained oriented in a peripheral ring just inside the membrane, in other cases, they appeared to fuse with one another, so that fewer than the normal haploid number (9) were present. This condition persisted until approximately 35 minutes after the beginning of treatment, at which time the egg nuclear membrane became much less distinct and the basophilic bodies within the membrane (representing either separate or fused chromosomes) were seen to be passing *through* the membrane. This process continued until by about 50 minutes after the beginning of treatment, the basophilic bodies were lying scattered in the egg cytoplasm, and the nuclear membrane had entirely disappeared. No asters or spindles were observed. No further changes were noted after this period, insofar as the chromosomes were concerned, and there was no subsequent development of the eggs.

The sperm nucleus remained unaffected by the podophyllin for about 60 to 70 minutes, after which time the same process of basophilic body extrusion was observed as occurred in the treated eggs.

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Shape changes in fertilized Chaetopterus eggs treated with podophyllin. CATHERINE HENLEY.

Fertilized Chaetopterus eggs treated with podophyllin by the methods described in the abstract by Henley and Costello above showed a number of shape changes which were remarkable simulations of such changes as observed in normal, untreated eggs of this form, despite the fact that maturation and cleavage spindles and asters were completely inhibited.

With the lower concentrations of podophyllin (particularly with the 0.0055 mg./cc. dosage), a semblance of polar body formation was often observed. This was characterized by the segregation of the entire egg nucleus into a large exovate (considerably larger than the normal polar bodies) which usually remained attached by a stalk to the egg cytoplasm. Such "pseudo-polar body formation" usually occurred at the same time as the formation of a normal first polar body in the controls, *i.e.*, approximately 11 minutes after insemination (6 minutes after the beginning of treatment). The pseudo-polar body was often resorbed into the egg cytoplasm within a period of about 10 minutes after its initial appearance, and there was usually no repetition of the event at the time of second polar body formation in the controls. In some eggs, exovates occurred in which no chromatin was present.

Concurrent in time with the characteristic pear and polar lobe stages of the control eggs, there were very normal-appearing similar stages in the experimental group. The polar lobes were subsequently resorbed by the eggs and no further development occurred. Cytological examination of such treated eggs showed that in many cases, at least, the shape changes could be correlated in time with the release of the basophilic bodies from the egg nucleus, as described by us above.

With the higher concentrations of podophyllin, exaggerated membrane elevation was observed, but this was not as pronounced as that seen after other experimental treatments.

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Examination of some D.P.N.- and T.P.N.-dependent dehydrogenase activity before and after relatively high doses of x-irradiation of Spisula eggs. E. KIVY-ROSENBERG, J. CASCARANO AND G. MERSON.

Work on the quantitation of dehydrogenase activity, using INT as hydrogen acceptor, in developmental stages of Spisula was reported by us earlier. The study of substrate-dependent dehydrogenase activity has been continued and extended somewhat.

Since the quantities of eggs are so limited in this form, gametes from 4–6 clams were pooled for each set of experiments and a microchemical technique employed. Samples of a 3.5% homogenate of both uninseminated and inseminated eggs were incubated aerobically at 37°–38° C. for one hour in a medium containing one of a series of substrates including succinate as well as some with DPN as cofactor, namely alpha glycerophosphate, glucose, glutamate, malate, lactate, beta hydroxybutyrate, ethanol and some with TPN as cofactor, namely malate, isocitrate, glucose-6-phosphate. Each set had appropriate controls.

The formazan was extracted and the amounts of reduced tetrazolium measured photocolorimetrically. The activity was expressed as micrograms of formazan per milligram of protein. The malate-dependent dehydrogenase activity was greatest and alpha glycerophosphate next in order for the DPN-dependent dehydrogenases, whereas isocitrate-dependent dehydrogenase activity was highest in the TPN-dependent dehydrogenases with malate and glucose-6-phosphate less active but still high.

To determine the effect of relatively high doses of x-irradiation upon their substrate-dependent dehydrogenase activity, 60,000 r was delivered to uninseminated and inseminated eggs. A covered plastic container of 30 ml. eggs in water was irradiated for 12′ 9″. The experimental series was made up of five situations: uninseminated controls, uninseminated x-rayed, inseminated controls, inseminated x-rayed and uninseminated x-rayed then inseminated.

Results seem to indicate that on the whole, x-irradiation increases some of the substrate-dependent dehydrogenase activity in both uninseminated and inseminated eggs: *e.g.* in the cases of the 5 most active dehydrogenases. This is not absolute or completely uniform for all egg pools however. It is less uniform in the TPN than in the DPN series. In the future, individual Spisula will be checked with the most effective substrates.

Extraction of egg jelly precipitating agents from Arbacia sperm. KURT KOHLER * AND CHARLES B. METZ.

Agents from sperm which precipitate egg jellies have previously been prepared by heating (Frank, 1939), freeze-thawing (Tyler, 1939) and mild acid extraction (Tyler and O'Melveny, 1941) of sperm. Such precipitating action has been attributed to a specific sperm receptor substance, antifertilizin. Upon further examination it is now found that frozen-thawed extracts of whole *Arbacia* sperm, isolated heads and isolated tails all precipitate egg jellies. These extracts were obtained as clear supernatants after 20 minutes' centrifugation at 3000 × g. At higher centrifugation such extracts yielded a sediment. With the formation of such sediment, jelly-precipitating action disappeared from the supernatant. This result was obtained in five independent extracts, one centrifuged at 7000 × g for two hours, two at 26,000 × g for thirty minutes and two at 100,000 × g for one hour. In two experiments, one at 26,000 × g, the other at 100,000 × g, such loss of activity from the supernatant was not demonstrated possibly because of resuspension of some of the sediment. In one experiment the 100,000 × g sediment was resuspended in sea water and found to precipitate jellies. In two experiments heating to 100° C. destroyed the egg jelly precipitating action of frozen-thawed extracts. These observations indicate that the jelly precipitating agent in frozen-thawed extracts is associated with particulate matter that coagulates upon heating.

On the other hand extracts prepared by heating sperm and centrifuging at 3000 × g failed to yield sediments after centrifugation at 100,000 × g. In one experiment aliquot extracts of a single sperm suspension were prepared by both heating and freeze-thawing. The egg jelly precipitating action of the frozen-thawed extract was removed but the heat extract remained active after centrifugation at 100,000 × g. These observations suggest that the jelly-precipitating agent in the heat extract is not associated with particulate material.

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The calcium-deficient uterus. H. A. KURIYAMA AND A. I. CSAPO.

Tension and action potentials (with intracellular microelectrodes) were recorded simultaneously *in vitro*. The uterine muscle of rabbits and rats was studied. Strips from the same uteri were examined at the same time using the "sucrose gap" method (see J. Marshall in this volume), to get an over-all picture of the relative changes in membrane potential and membrane activity. Spontaneous and electrically- or pharmacologically-induced mechanical activity were also recorded from an additional pair of strips of the same uterus, to determine electrical excitability, threshold, maximum tetanus, effective drug concentration, long range behavior, etc.

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If a parturient uterus is given a low concentration of oxytocin (for example 5 μU/ml.) and the early phase of membrane and myoplasmic activity is recorded, one can observe the one-to-one ratio between these two events. One action potential triggers a twitch, a number of them with low frequency an incomplete tetanus, whereas a train of discharges of appropriate frequency gives rise to a fused tetanus. An oxytocin concentration of 50 μU/ml. maintains for hours regular and maximal electrical and mechanical activity. The membrane potential fluctuates between the trains of discharges and each train is usually preceded by depolarization.

If the uterus in a steady state of maximum activity is rinsed repeatedly with Ca-free Krebs the membrane potential drops below 30 mV, the spikes become smaller, fewer and less frequent, and in 15–30 minutes activity ceases altogether. In this state of Ca deficiency the oxytocin effect is reduced to an abortive contracture. A threshold concentration of Ca (for example 0.3 mM/l.) increases the membrane potential above 30 mV and small spikes appear. With this change pharmacological reactivity returns, but in this condition oxytocin increases the membrane potential slightly, giving rise to large action potentials forming regular trains. As before the membrane potential fluctuates between trains of discharges and each train triggers a myoplasmic response. Thus, depending on its initial value the membrane potential is adjusted by oxytocin and is kept in a critical range where spontaneous activity is maximal.

Progesterone *in vitro* (10 μg./ml.) reduces the amplitude of the action potentials. Tension is also reduced, whether spontaneous, electrically- or oxytocin-induced.

The "evolution" of membrane and myoplasmic activity of uterine muscle. H. A. KURIYAMA AND A. I. CSAPO.

Tension and action potentials (with intracellular microelectrodes) were recorded simultaneously *in vitro*. Spontaneous electrical and mechanical activity of the uterus was studied during sexual maturation, pregnancy and parturition. In parallel experiments, isometric tension alone was recorded, and electrical excitability, threshold, maximum tetanus, effective drug concentration, long range behavior, etc., were studied.

The uterus of immature rabbits (4 months old or younger) has a membrane potential less than 30 mV. The spontaneous membrane discharges are very small and irregular, triggering irregular or no mechanical activity. Oxytocin does not improve upon such activity but induces a slight contracture. Incomplete estrogen stimulation slightly increases the membrane potential, spike amplitude and regularity. But tension remains small, irregular and of high frequency, with no adequate resting periods between activity. Oxytocin only exaggerates this contracture tendency.

Prolonged estrogen treatment brings the membrane potential up to 45 mV. The amplitude of the action potential increases, forming more or less regular trains, which trigger more synchronous mechanical activity. Oxytocin improves upon the regularity of the trains of discharges, increases their frequency and duration, thereby increasing mechanical activity. During the third trimester of pregnancy the membrane potential increases to a peak value of about 60 mV at placental sites. During this period regular trains of action potentials are not observed at placental portions, and irregular trains are only seen after several hours of perfusion, when they trigger asynchronous activity. Until the thirtieth day of gestation the rabbit uterus does not respond to 500 mU/ml. oxytocin, whereas the parturient uterus is affected by 0.5 μU/ml.

Delivery occurs at a membrane potential of about 50 mV. Immediately after parturition the uterus exhibits very infrequent spontaneous activity and only about 12 hours post partum does activity reach a high frequency. At this time the membrane potential dropped below 50 mV. The climax in the evolution of membrane and myoplasmic activity seems to be reached, therefore, a few hours after delivery. This climax can be effectively precipitated earlier by oxytocin. The membrane potential is close to 40 mV, 32 hours after delivery. Spontaneous membrane and myoplasmic activity then become somewhat irregular and tension begins to decrease.

It appears that regular phasic mechanical activity is closely linked to regular trains of action potentials of appropriate amplitude and frequency. Such membrane activity is induced by estrogen and if present may be improved upon by oxytocin. Progesterone, on the other hand, suspends such activity.

Glucose-6-phosphatase content of toadfish islet tissue. ARNOLD LAZAROW, PAULA MAKINEN AND S. J. COOPERSTEIN.

Histochemical studies by other investigators have suggested that the beta cells of the islets of Langerhans are rich in glucose-6-phosphatase. This is of particular interest in view of the known relationship of glucose to the insulin-release mechanism. The method for the direct chemical analysis of this enzyme has been adapted to a micro scale and applied to toadfish islet tissue. In this species the islet tissue is separated from the exocrine pancreas and concentrated into a segregated body known as the principal islet.

The liberation of inorganic phosphate from glucose-6-phosphate was measured at pH 6.5 following a 15-minute incubation period at 37° C. The reaction mixture contained 2–5 μl. of a 1 : 5 islet homogenate (or a 1 : 40 liver homogenate), 0.04 *M* glucose-6-phosphate, 0.007 *M* histidine, and 0.001 *M* Versene in a total volume of 30 μl. Comparative studies were also carried out in which glycerophosphate was substituted for glucose-6-phosphate. The results were expressed as mμg. of phosphorus liberated per μg. of tissue in 15 minutes and the figures represent the average of 5 separate experiments with each tissue and each substrate.

Liver liberated 5.7 mμg. of phosphorus when glucose-6-phosphate was the substrate, as compared to 0.51 mμg. when glycerophosphate was used. By contrast islet tissue liberated 0.36 and 0.35 mμg. from glucose-6-phosphate and glycerophosphate, respectively. These data suggest that, in contrast to liver, the liberation of inorganic phosphate from glucose-6-phosphate by toadfish islet tissue may be due to the action of a non-specific phosphatase rather than to the presence of a specific glucose-6-phosphatase in this tissue.

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Leucothrix mucor. RALPH A. LEWIN.

L. mucor Oersted is possibly a colorless member of the Rivulariaceae (Cyanophyceae), hitherto isolated in Europe and western North America. Thirty-six isolates were obtained from the surface of algae, especially *Callithamnion* sp., along shores in the region of Woods Hole and Penikese Island. Pure cultures were established by streaking on sea water + Tryptone (0.2%) + agar (1.0%) medium. Streptomycin could not be used in the purification, since 25 mg./l. proved inhibitory to *Leucothrix*. The strains isolated differ only in minor characters, *e.g.*, degree of fragmentation and gonidia production, but these differences persist in culture.

By varying the components of synthetic sea-water media, 3.0% NaCl was found optimum, though growth occurred in concentrations down to 0.4%. Both K and Ca are essential. The following basal medium was found satisfactory for aerobic growth when supplemented with suitable N and C sources: NaCl, 30.0 g./l.; KCl, 1.0 g./l.; $CaCl_2 \cdot 2\ H_2O$, 0.1 g./l.; $MgSO_4 \cdot 7\ H_2O$, 0.1 g./l.; K_2HPO_4, 0.1 g./l.; "Tris" buffer, 1.0 g./l.; Fe and other trace elements; pH 7.7. In 48 hours at 25° C. dense flocculent growth was obtained with Tryptone (5.0 g./l.) or Na glutamate (10.0 g./l.). In basal medium with optimum concentrations of Na lactate (10.0 g./l.) and NH_4Cl (3.0 g./l.), growth was less dense, but no further stimulation could be promoted by the addition of yeast extract and cobalamin. The following compounds, tested in auxanograms or at various concentrations in liquid media, could not be used as sources of C for growth: glycerol, mannitol; arabinose, fructose, fucose, galactose, glucose, maltose, mannose, rhamnose, sucrose; acetate, citrate, fumarate, glycollate, malate, succinate; asparagine.

The effect of indole acetic acid and serotonin on the calcium^{45} content of leaves of the fresh-water plants, Potamogeton crispus (L.) and Elodea canadensis (Michx.). BENJAMIN LOWENHAUPT.

Leaves of *Elodea canadensis* (Michx.) and *Potamogeton crispus* (L.) immersed in $Ca^{45}Cl_2$ solution acquire radioactivity which reaches an equilibrium or steady-state level in a few hours. Previous work has demonstrated that leaves thus prepared are useful in studies of calcium transport and equilibrium. Indole acetic acid and serotonin, it was found, release some of the calcium^{45} radioactivity from such leaves into their ambient solution. Effects of light and pH are significant, but have not yet been worked out.

This study was initiated in collaboration with Dr. Richard Klein at the New York Botanical Garden.

The effects of calcium deficiency and oxytocin on the membrane activity of uterine muscle, as measured by the "Sucrose Gap" method. J. M. MARSHALL AND A. I. CSAPO.

It is possible to measure, with external electrodes, membrane resting and action potentials from a strip of muscle, assuming that the muscle acts as a core conductor and that the short-circuiting between the electrodes is negligible. The latter is accomplished by increasing the external resistance of the muscle between the electrodes by replacing the interstitial fluid with sucrose. This method, the "Sucrose Gap," permits a continuous recording of changes in membrane potential and in rates of action potential discharge for a period of several hours.

Strips of uterine muscle from rats and rabbits were used. Portions of the same uteri were studied with microelectrodes under identical conditions (Kuriyama and Csapo, this volume). There was a close correlation between the results of the two methods.

The membrane potential of the parturient uterus underwent rhythmical cycles of depolarization and repolarization, the frequency of which was related to the mechanical contractions. During depolarization action potentials usually appeared. If oxytocin was applied during the quiescent phase of the cycle (repolarization and no action potentials), the membrane would immediately depolarize and action potentials would arise. When the parturient uterus was repeatedly perfused with a solution containing no calcium, the membrane depolarized about 20 mv. and all action potentials disappeared. If a small amount of calcium was added to the perfusion fluid (0.25 mM/l) the membrane potential increased somewhat although no action potentials appeared. The addition of oxytocin under these conditions increased the membrane potential to a level where action potentials appeared.

When a strip of muscle from a parturient uterus was perfused, *in vitro,* with a solution containing progesterone (20 μg./ml.) the fluctuations of the membrane potential and rhythmical discharges of action potentials were abolished and the membrane potential increased slightly. Oxytocin under these conditions was ineffective.

Supravital dye studies on isolated cells of frog renal adenocarcinoma. G. M. MATEYKO.

Dissociated, living cells of the frog tumor (Lucké renal adenocarcinoma) were immersed in supravital dye solutions of the following dye series: acridines, quinone-imine dyes, mono- and dis-azo dyes. Characteristic neutral red-staining granules of these tumor cells appear to be basophilic (RNA) components. Of particular interest were the thiazine dyes, methylene blue, methylene violet, Bernthsen (deaminated methylene blue), thionine, and toluidine blue 0, which showed initially, pale blue nucleoli within colorless nuclei, lavender cytoplasm with many coarse, irregular purple granules, but unstained spherical (neutral lipid) granules. This metachromasia is evidence of a viable cell population, the selective dye accumulation being dependent upon an active cellular process. Deep orthochromatic staining of the nucleus and cytoplasm is characteristic of a moribund population. Acriflavine and acridine yellow, however, exhibit an intensive and persistent nuclear staining in living cells (even without ultraviolet illumination), whereas with most dyes, nuclear staining is indicative of cell death. Accordingly, selective dye pickup and subcellular segregation, in contrast to diffuse orthochromasia of necrotic cells, are characteristic of a living cell population. Viability may be established, therefore, by the thiazine dyes, as well as neutral red, and vital red.

Dye accumulations were strikingly similar immediately after cell separation, and after storage at 4° C. for 24 or 48 hours, even in tumors from different animals. In contrast to the above, the dye patterns of normal animals show little vital metachromasia, especially in the reduction or lack of coarse stained cytoplasmic granules.

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The localization of some dehydrogenases in Echinarachnius parma. GERALD MERSON.

It had been demonstrated that malic and succinic dehydrogenase systems could be isolated during the development of Arbacia. Work in this form was done on homogenates with TTC (triphenyl tetrazolium chloride) as the hydrogen acceptor. The present work was carried out histochemically on *Echinarachnius parma,* using a newer and a more rapidly reacting tetrazolium salt, INT (2-p-iodophenyl-3-nitrophenyl-5-phenyl tetrazolium chloride) in an effort to localize the dehydrogenases above. Toward this end ten stages of development were utilized: unfertilized, and fertilized egg, 8-cell, early and late blastula and gastrula, and early and late pluteus stages. In each case about 60 specimens were incubated in one of five media: (A) sodium malate (.2 *M* made in Ca-free sea water) with DPN and INT; (B) sodium succinate (.05 *M* made in Ca-free sea water) and INT; (C) Ca-free sea water and INT; (D) Ca-free sea water; and (E) untreated sea water. The final dilution of INT was about 0.0125%. Ca-free sea water was used for improved solubility of INT. The embryos were incubated anaerobically for 1½ hours at water table temperature (19.5° C. ± 0.5° C.) and observations made within the following half hour.

One can demonstrate a clear-cut precipitate of reduced INT(formazan)1 starting at the early gastrula stage. This activity is seen in the succinic and malic dehydrogenases. It is localized in the two areas of developing primary mesenchyme. In the mid-late gastrulae, this activity is extended to include a semicircle of cells which joins the two isolated plaques. During the pluteus stage, this activity is seen in all the arms and along the margins between the oral arms. Embryos incubated in the Ca-free and INT control (C) demonstrate only a mild diffuse pink color. All media permitted organisms to remain viable although development did not progress in any except the untreated sea water. Thus it appears that the primary mesenchyme is a site of malic- and succinic-dependent dehydrogenase activity.

Early responses of alloxanized toadfish. P. F. NACE, C. YIP, I. SILKOVSKIS, M. MOULE AND J. MOULE.

Beginning May 22, at sea water temperature of 14° C., adult toadfish of both sexes were injected with alloxan, 16% aqueous, at 700 mg./kg., through the heart. At intervals 1 minute to 23 days after treatment, blood samples were taken and fish were sacrificed for histological study. About 500 fish were studied by modified Folin (Murrell-Nace) and Glucostat blood sugar procedures, with fixation, after sacrifice, of principal pancreatic islet, liver, kidney (with interrenal) and brain (with pituitary). A smaller number of treated and control fish were injected with tritiated thymidine or with cystine labelled with sulfur-35. Pooled samples of blood of treated and control fish were studied by electrophoresis.

Egg-bearing females did not exhibit the "hyperglycemia" characteristic of males in the breeding season or of both sexes later in summer, after spawning and after water temperatures had exceeded 18° C. Maintenance of spawned females in water cooled to 15° C. did not restore resistance to alloxan, nor did it restore the 30–35 mg.% intact blood sugar levels found July 13, at 20° C., toward the 65 mg.% values found at 14° C. in May. Daily treatment with estrogen for 7 days did not change blood sugar level, but thyroxine increased response to lower doses of alloxan. For intact fish, and for fish sampled more than 6 hours after treatment, Folin and Glucostat values were comparable. However, the Folin "hyperglycemia" seen 1 minute to 6 hours after treatment was not reflected in parallel Glucostat determinations. Electrophoresis revealed no substance in experimental blood implicable in this divergence. The absence of the response in egg-bearing females militates against the implication of alloxan, which is a Folin-detectable reducing substance.

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Phosphoprotein phosphatase in the ovary of R. pipiens. SYLVAN NASS.

Differential centrifugation of ovarian homogenates of *R. pipiens* indicates that phosphoprotein phosphatase is located primarily in the small yolk platelets. Most of the small endogenous activity found in clean "large" yolk at the enzyme's optimum pH (5.0) can be removed by solubilizing in 0.4 *M* NaCl and re-precipitating by dialysis against 0.1 *M* NaCl or distilled water. Attempts to extract the enzyme from the small platelets have been only partially successful. After 0.4 *M* NaCl extraction the extracts and residues contain similar activities. This is a result of either or both of two factors: (a) the small yolk platelets are less readily soluble in high ionic strengths than are the larger platelets; (b) the enzyme is rapidly adsorbed on pigment granules. Uncontaminated pigment granules have no PPPase activity. Attempts to extract the enzyme from the total homogenate after removal of the enzymatically inactive soluble proteins were discontinued because the extracted proteins were incompletely re-precipitated upon dialysis. By contrast, clean yolk platelets, when extracted are completely re-precipitated. Approximately 10% of the total protein phosphorus of phosvitin, casein and yolk is hydrolyzable by the enzyme. Yolk heated to 80° C., β-glycerophosphate, ATP and RNA were not attacked by the enzyme. The addition of 1% RNA to a phosvitin-enzyme preparation prevented inorganic phosphate release, while with ATP there was a slight inhibition. Inhibition studies indicate that the enzyme has somewhat different properties from those published for mammalian PPPases. In agreement with reports on mouse liver PPPase, frog egg PPPase is most strongly inhibited by molybdate (50% at 10^{-4} *M*). Cu^{++}, Hg^{++} and Mn^{++} inhibited 100% at 10^{-2} *M*, 20% at 10^{-4} *M*. However, Ca^{++} and Mg^{++} had very little effect at 10^{-2} *M*. Thioglycollic acid, desoxycholate, ascorbic acid, hydrogen peroxide, oxalate, and phenanthroline were without effect although both activating and inhibiting effects were found with these compounds by other workers on PPPases of different origins. Additional studies are necessary before any interpretation of these results can be made.

Uptake of Zn^{65} and primary productivity in marine benthic algae. EUGENE P. ODUM AND ROGER W. BACHMANN.

The rate of uptake of zinc-65 from normal sea water and the primary production of ten species of macroscopic benthic algae were measured in light and dark bottles suspended in a running sea-water aquarium under constant light and temperature conditions. Uptake and production of one species (*Chaetomorpha Linum*) were also measured in bottles suspended at different depths in a marine pond in order to achieve a gradient of light intensities. Approximately 3 μc of Zn^{65} were added to each bottle containing 250 ml. of sea water and approximately 0.5 gm. dry weight of algae. Definite uptake occurred in the light, but no appreciable uptake occurred in the dark. The rate of uptake in the light was proportional to gross oxygen production which varied with the species and the light intensity. Species with high surface-to-volume ratios exhibited more rapid uptake of zinc and greater gross oxygen production per gram biomass than species with low surface-to-volume ratios. Uptake of Zn^{65} apparently depends on photosynthetic activity since no uptake occurred in the dark and the rate in the light was independent of the concentration of the isotope or carrier added to the water. Consequently, Zn^{65} has possibilities as a tool for the measurement of primary productivity.

Protoplasmic contraction and cell wall swelling in a marine alga. W. J. V. OSTERHOUT.

A marine alga, *Chaetomorpha Linum* Kützing, was used. These cells were smaller, younger and less resistant to external changes than the cells used in previous years, which may be responsible for the difference in the results.

(I) A string of living cells was transferred at room temperature (25° C.) from sea water to distilled water. A crescent-shaped swelling appeared on the surface of the cell wall and the string of cells moved by irregularly bending until one spot on each cell burst. The protoplasmic contents rapidly flowed out, forming a green mass outside at the point of exit, and the cell became practically devoid of protoplasm. When a solution of 0.05 *M* NaH_2PO_4 at pH 4.5 was used the same results were obtained except that the viscosity of the protoplasmic mass was so much greater that it formed a thick plug at the cell wall opening which prevented further exit of the protoplasm. The protoplasm remaining inside the cell markedly contracted after a few minutes, while the cell wall remained swollen. These results indicate a difference in the mechanisms controlling the cell wall and the protoplasm. With the cells used in previous years there were no changes even two hours after the cells were transferred from sea water to distilled water. As above indicated this difference may be due to difference in the age and in the previous history of the cells.

(II) A string of living cells was heated in sea water at 50° C. for five minutes until the cells were injured and acid fuchsin penetrated the cells. The string of cells was transferred to sea water at room temperature (25° C.) and after thirty minutes it was placed in distilled water at 25° C. Swelling of the cell wall occurred but there was no bursting. The protoplasm contracted after about thirty minutes. In 0.05 *M* solution of phosphate buffer mixture at pH 6 the protoplasmic contraction occurred more rapidly than in distilled water but the difference in the rate was not so marked as in the case described in 1955 in this journal. This difference in the results may be due to the difference in the age and in the previous history of the cells. If 0.05 *M* solution of NaH_2PO_4 at pH 4.5 was used the protoplasmic contraction occurred more rapidly than with a solution of phosphate buffer mixture at pH 6.

Water relations in injury to Nitella cells. W. J. V. OSTERHOUT.

Single cells of *Nitella flexilis* about seven centimeters long were used. A cell was divided into two parts, A and W, by a vaseline seal. The movement of liquid inside the cell was observed by following the movement of suspended particles. If water was placed at A and at W there were no changes. But when water at A was replaced by a toxic concentration (3 *M*) of ethyl alcohol solution a striking series of changes occurred. (1) A rush of liquid inside the cell from W to A occurred. This was due to the higher osmotic pressure of alcohol at A which extracted the water from the cell at A while the water entered the cell at W. Suspended particles and dissolved substances were carried with the liquid from W to A and accumulated at A where they could not come out of the cell. The rush from W to A soon stopped when the osmotic pressure inside the cell at A increased so that water ceased to go out of the cell rapidly at A. (2) Then the reverse rush from A to W began. This was due to the fact that alcohol now entered the cell faster at A than water came out of the cell at A. This rush soon stopped because the osmotic pressure of the cell at W increased when dissolved substances inside the cell accumulated at W since they could not come out of the cell along with the liquid at W. (3) Then a more violent rush began from W to A. This was due to increase in permeability of the cell to substances inside the cell at A caused by injury from alcohol at A. The contents poured out at A from the cell which collapsed and died.

These changes were seen when a stained cell was used. Normally, brilliant cresyl blue accumulated uniformly in the vacuole because in the form of ions the dye could not come out of the cell. (1) In the first rush the dye was seen moving from W to A where it collected. (2) In the second rush the dye began to move from A to W. (3) In the third rush the dye came out of the cell at A because the permeability to dye ions increased.

These results may be due to changes in concentration only in the immediate vicinity of one or both of the protoplasmic membranes (plasmalemma and tonoplast).

If a non-toxic solution of ethyl alcohol (0.5 *M*) was used another process occurred as reported in 1950 in the Journal of General Physiology.

A study of the soluble proteins of the adult squid lens. JOHN PAPACONSTANTINOU.

The soluble proteins from the lens of the adult squid, *Loligo pealeii,* were fractionated by use of the anion exchanger, diethylaminoethyl cellulose (DEAE). The fibers from the peripheral and core regions of the lens were homogenized separately in .005 *M* phosphate buffer, pH 7 and the clear supernatant of this homogenate was applied to a DEAE column. The protein was eluted from the column by a stepwise addition of phosphate buffers in which there was a simultaneous increase in ionic strength and decrease in pH. Aliquots of 10 ml. were collected and analyzed for protein concentration. In all experiments 92–95% of the protein applied to the column was recovered. Analysis of the elution diagrams showed that 85% of the soluble protein from the peripheral lens fiber homogenate and 93% of the soluble protein from the core lens homogenate were eluted by the starting buffer, *i.e.,* .005 *M* phosphate buffer, pH 7. To determine whether this "break through" protein consisted of several protein components, antibodies to the peripheral and core homogenates were prepared and each 10-ml. fraction in the "break through" region of the elution pattern was tested by the agar diffusion technique. It was found that only one very heavy band was formed when aliquots of the 10-ml. fractions were permitted to diffuse against antisera to the peripheral and to the core lens proteins. The combined DEAE fractionation and agar diffusion experiments indicate that there may be only one major soluble protein in the peripheral and core fibers of the squid lens. Further analysis by electrophoresis and ultracentrifugation will be done to support the data presented above.

A calculator to aid in the correction of astigmatism in electron microscopes. DELBERT E. PHILPOTT.

A circular calculator for correction of astigmatism has been made of plastic to aid in teaching and to speed up the correction process. A 5-inch clear plastic disk is placed over a 360° segmented circle and attached in such a way that it is free to rotate. The positions of the eight iron screws located in the objective pole piece are plotted around the periphery of the circle. An inked line is drawn through the center of the plastic disk with an arrow pointing out at each end, the tips terminating on the edge of the segmented circle. This line is labeled "astigmatism produced." To make this calculator, the angle between screw direction and astigmatism is now determined in the regular way by sufficiently moving one pair of screws to produce a large increase in astigmatism. When this angle has been determined, the "astigmatism produced" line is set in the direction of the astigmatism and the screw direction which produced this astigmatism is plotted on the surface on the plastic in the form of arrows pointing inward. Using this calculator to correct astigmatism, set the "astigmatism produced" line at 90° to the astigmatism which is present in the microscope. The screws which must be turned in for correction are now read directly below the "screw direction" arrows. This system helps to obviate such things as uneven screw tips and screw wobble which can cause the astigmatism to change direction inconsistently causing confusion in the compensation. In this laboratory the screws are used to fully compensate the electron microscope and the electrostatic compensator is used to correct any astigmatism produced by the objective aperture. Otherwise the two vectors must be corrected at one time. Hence, two systems of correction again simplify the operation of the electron microscope. The increased visualization is also a distinct aid in teaching. The above procedure has been applied to electrostatic compensation with equal results.

An electron microscopic study of the sperm of Limulus polyphemus. DELBERT E. PHILPOTT AND WILLIAM N. SHAW.

The sperm of *Limulus polyphemus* was fixed in osmic acid, embedded in methacrylate and sectioned for electron microscopy. The anterior end of the sperm head consists of an acrosome cap, and a "small" axial body (a larger one was described in oyster sperm). An axial core traverses the head centrally from the small axial body to the region of the centriole in the distal end of the head. Immediately adjacent to the region of the centriole the axial core, or its continuation, bends laterally to the outer surface of the head and appears to be continuous with the coiled structure which forms approximately six closely adhering spiral turns around the base of the head. This posterior spiral seems to originate from the axial core but the axial core is hollow and the spiral is uniformly osmiophilic and apparently solid. Fitting up against this spiral coil and covering the posterior end of the head are numerous small mitochondria.

Normally the sperm flagellum arises from a basal structure which is made up of two centrioles and the origins of the filaments comprising the tail. A centriole region has been observed in Limulus sperm, but two distinct centrioles have not been resolved.

The tail proper is composed of a pair of central filaments and an outer ring of nine pairs of filaments. The central filaments stop just before they reach the attachment to the head; the outer filaments continue up into the head. The result is a weak point in the attachment region and any change in osmotic pressure breaks the tail at this point.

The entire sperm head is covered by an outer membrane which continues posteriorly beyond the mitochondrial region of the midpiece for a distance of .8 μ and then invaginates for the same distance to the proximal region of the tail. The posterior extension thus formed contains a spiral or possibly metameric structure which probably serves as a stiffening agent for the region of the tail attachment. The discharged sperm were examined for acrosomal filaments and several sections showed this filament to extend from the small axial body directly through the acrosome cap and out into free space. Further work is being continued on this material.

Chemical excitation of presynaptic terminals at lobster neuromuscular junctions. J. P. Reuben, F. Bergmann and H. Grundfest.

Although picrotoxin selectively blocks inhibitory postsynaptic membrane of decapod muscles, it also sensitizes terminals of both excitatory and inhibitory axons to other drugs: *e.g.*, beta-phenethylamine, serotonin, tetraethyl- and tetrabutylamine, and veratrine. Spontaneously, or after a single brief stimulus, an axon fires repetitively at rates up to 50/sec., often for several seconds. The "antidromic" impulses thus produced propagate to stimulate other muscles supplied by the axon. The repetitive activity is shortened, or stopped by GABA. Then, addition of more picrotoxin restores it, the interaction apparently being due to a competitive antagonism of the effects of picrotoxin and GABA. Several varieties of data localize the site of these actions to the nerve terminals. Applied to the axon itself, the drugs prolong the spike, or block it, the combination picrotoxin-serotonin being about equally effective as the picrotoxin-phenethylamine combination. Nor is the muscle directly involved in the drug-induced repetitive activity of the axon. The individual axons fire at different rates and asynchronously, repetitive activity in one axon not affecting that of the other in the case of the stretcher innervation. When muscles are supplied with more than one excitatory axon the thresholds of the drug effects are different. The membrane resistance and resting potential of the muscle fibers are not changed by the drugs prior to the onset of the repetitive firing of the axons. The electrical excitability of the muscle fibers may be eliminated, as by selective depolarization by glutamate, without affecting the axonal discharges. Therefore it seems likely that the picrotoxin sensitizes the nerve terminals to the other drugs which then depolarize the terminals. The resulting "generator" potential would then tend to fire the axons. The reversal of the picrotoxin action by GABA forms a striking parallel to the antagonism of these two substances on lobster neuromuscular synaptic membrane or axodendritic synapses of the cat cortex. However, the nature of the action is different in each case.

Anomalous current-voltage relation induced by hyperpolarization of lobster muscle fibers. J. P. Reuben, R. Werman and H. Grundfest.

Intracellularly applied hyperpolarizing pulses cause a discontinuous change in the membrane potential. At a threshold of about 70 mv hyperpolarization, and while the current remains constant, a "response" begins with variable latency, rising slowly to about 200 mv hyperpolarization. Stronger currents shorten the latency. The response can be induced by adding a brief anodal pulse to a subthreshold current. It can be abolished by cathodal pulses in all-or-none fashion, indicating a range of forbidden potential between threshold and response peak. The response is variable in form and duration. Sometimes it persists, with or without small oscillations, throughout the applied current, or it may terminate earlier, the potential returning to approximately the threshold value. In the latter case, several more or less identical responses may occur. Measured by amplitudes of applied brief pulses, or of evoked epsp's, the membrane resistance at the response peak is 3 to 4 times higher than at the threshold. Summated ipsp's restore the membrane potential approximately to the Cl equilibrium potential. Probably, therefore, neither synaptic membrane nor Cl-permeability are involved in the response. Addition of Ba, or of high Ca, which augments membrane resistance by blocking K-permeability (Werman and Grundfest, below; Reuben, unpublished), abolishes the response, as does 2 × K; the divalent ions by raising the membrane resistance as high as or higher than that at the response peak, while K raises the threshold. Thus, the response is probably caused by an abrupt, temporary decrease of K-permeability. At threshold currents the resistance then shifts discontinuously from its resting value to a higher one which can also be induced by divalent cations, particularly Ba and Sr. The "response," therefore, is an augmented IR drop, not a shift in membrane electrode properties. The change evoked by threshold currents is local and its electrotonic spread has two components; the potential during the "response" is attenuated less, but is prolonged more than is the potential below threshold, since the space constant is increased in correlation with the higher membrane resistance. A similar anomalous effect occurs in electroplaques of *G. carapo* (Bennett and Grundfest, unpublished) and may be a phenomenon common among excitable membranes.

The effect of sulfhydryl compounds on the colloidal state of the cytoplasm of Arbacia eggs. HOWARD ROTHSTEIN.

Earlier work of Rapkine, Mazia and Heilbrunn has clearly indicated the importance of the SH–SS transformation for the colloidal changes that occur in the cytoplasm during the course of the mitotic process. An attempt has been made further to elucidate the relation between the SH–SS equilibrium and the sol-gel transformation.

Unfertilized eggs of *Arbacia punctulata* were exposed for 30 minutes to 0.075 *M* solutions of mercaptoethanol and 0.085 *M* solutions of thioglycolate. In both cases the viscosity of the protoplasm increased significantly.

As Mazia has shown, mercaptoethanol inhibits cleavage in the Dendraster and Stronglocentrotus eggs. This has now been shown also to be true for the Arbacia egg. In addition, it was demonstrated that this inhibition is due, at least in part, to a prevention of the reversal of the mitotic gelation. That is to say, the viscosity of the cytoplasm increases after 20 minutes (at 21° C.) just as it does normally, but it never decreases again as it does in the controls, and the eggs cannot divide.

SH inhibitors were also employed. Mercuric chloride (10^{-5} *M*), 10^{-4} *M* iodoacetamide and 10^{-3} *M* p-chlorophenylmercurisulfonic acid all inhibit cleavage, seemingly through a similar colloidal effect, *i.e.*, prolongation of the mitotic gelation. Wilson and Heilbrunn have shown this for p-chloromercuribenzoic acid as well.

O-iodosobenzoic acid (10^{-3} *M*) was also employed, but no effect could be detected, either on cleavage or the attendant viscosity alterations. Possibly this compound is reduced in a more or less non-specific manner within the cell. It should be noted also that these compounds do not affect the viscosity of the unfertilized egg.

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Studies by filter paper electrophoresis of the hormones controlling color changes in the shrimp Crangon. MURIEL I. SANDEEN AND MILTON FINGERMAN.

Extracts of circumesophageal connectives with the tritocerebral commissure attached of *Crangon* were applied, using hot air, to one-half inch wide Whatman No. 1 filter paper strips. Each strip was submitted to electrophoresis at 500 V. and 0.1–0.2 mA. for a given period of time using citrate-phosphate buffer at pH 5.2 or a borate buffer at either pH 7.8 or pH 9.0. Each strip was cut into desired sections which were washed for 30 minutes with sea water. Eyestalkless *Crangon* were used as assay animals for these sea-water extracts. At each pH used both the tail-darkening hormone and the body-lightening hormone were electropositive. Using pH 9.0 electrophoresis was carried out for 2, 3, 5 and 16 hours. In each case the greatest amount of tail-darkening hormone and some body-lightening hormone were recovered from the ¼-inch origin while the greatest amount of body-lightening hormone was recovered from the first inch toward the cathode. After 5 and 16 hours significant amounts of body-lightening hormone with little or no tail-darkening hormone were recovered from the second inch toward the cathode. No hormone was ever recovered beyond 3 inches from the origin, whether or not the extract was boiled prior to application to the paper. Similar experiments were carried out using extracts of the eyestalks submitted to electrophoresis for 5 hours. To detect the antagonistic tail-lightening activity one-eyed *Crangon* adapted to a black background were used as assay animals. In 4 out of 7 cases, regardless of the pH maximum, tail-lightening activity was recovered from the first inch toward the cathode. In the other cases maximum activity was in the second inch.

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Intra-specific reaggregations in tunicates labeled with tritiated thymidine. Sister Florence Marie Scott and Joseph E. Schuh, S.J.

Cultures of *Amaroecium constellatum*, in early stages of metamorphosis, were exposed to either 1 μC/1 ml. or 5 μC/1 ml. of tritiated thymidine in standing sea water for two hours. The cultures were refreshed in running sea water for three hours and then immersed in tritiated thymidine for another period of eight hours. Cultures from the same shedding were kept in running sea water for a corresponding period of time. Late in metamorphosis (12 to 18 hours), when the young adult organization is established, the following combinations were made: 1) two tritiated branchial baskets and one non-tritiated abdomen; 2) two non-tritiated branchial baskets and one tritiated abdomen. The three fragments were ejected from their tunics and inserted into the evacuated tunic of a two-week-old Amaroecium. They were macerated together in the host tunic, pressed into place by plugs of tunicin and kept in running sea water. At critical stages through five days of development, radioautographs were prepared from serial sections.

Two types of aggregates develop from the combinations in which an epidermal envelope is reconstituted to enclose the fragments: 1) single individuals resembling Siamese twins, with two branchial baskets variously joined to a common abdomen; 2) twins of unequal size; one the product of a fused branchial basket and abdomen, the other a regenerated zooid from the second branchial basket. In both types, each fragment reconstitutes itself into an integrated branchial basket or abdomen. From a cursory examination of the radioautographs, it is clear that the reconstituted members then unite to form functioning complex zooids in the cases of the Siamese twins, and typical zooids in the cases of the double fusions. The areas of junction between tritiated and non-tritiated fragments are easily traced in the radioautographs which provide a precise and diagnostic means of identifying and following tissues in their reconstituting activities and in their final reaggregation into organisms.

Paper chromatography of pericardial organs of crayfish. Thomas Smyth, Jr.

Heartbeat in crustaceans is initiated by an intrinsic ganglionic pacemaker, modulated by extrinsic acceleratory and inhibitory nerves and by "neurosecretion" from pericardial organs lying outside the ostia. It has been reported that the pericardial secretion is mainly excitatory, but inhibition was found on occasion. In order to isolate the excitatory substance and possibly unmask an inhibitory one, paper chromatography of crayfish pericardial organs was tried. Only excitatory material was found.

This material is soluble in water and 50% ethanol, is not extracted by ether, withstands boiling and is non-volatile. Activity was lost by desalting with Permutit-50 and on storage of boiled aqueous extracts for two weeks in the deep freeze.

Hot water or hot 50% ethanol extracts of pericardial organs were spotted directly on washed Whatman # 1 filter paper. Extracts equivalent to the pericardial organs of two crayfish were used for each paper. Ascending methanol: water: pyridine (80:20:4) was the best solvent system tried. After drying, the papers were cut in strips, eluted with water, extracted with ether, lyophilized and redissolved in Tris-buffered van Harreveld's solution for assay on crayfish hearts. Activity resembling that of the crude extract was found only in strips corresponding to an R_f of 0.68. Since activity steadily disappeared, highest activity was recovered when the entire process was conducted without pauses between the various operations. Recovery was not improved by developing in a nitrogen atmosphere or by chromatogramming with added ascorbic acid. The active region was not obviously ultraviolet fluorescent or absorbing, did not react with Ehrlich's or Dragendorf's reagent, stained lightly with ninhydrin and with ammoniacal silver nitrate.

The excitatory substance is apparently not serotonin, as this had an R_f value of 0.20 (agreeing with Carlisle, 1956). Only a fraction of the activity was lost on boiling in normal HCl or on digestion with trypsin.

Relation of ultraviolet-ray dosage to modifications in fertilization and early development of Arbacia. Carl Caskey Speidel and Ralph Holt Cheney.

Systematic studies were made of the modifications in fertilization and early development of the sea urchin induced by ultraviolet radiation. The experimental material (eggs, sperm, embryos) to be irradiated was exposed to ultraviolet rays of 2537 Å wave-length from a lamp 6 inches away. Exposures ranged from 2 seconds to 48 minutes. The principal studies were made on unfertilized eggs unilaterally exposed for periods of 0.25, 0.5, 1, 2, 4 and 8 minutes, respectively. These were fertilized with normal sperm and kept protected from light. By special techniques, samples from each series were examined and compared continuously for effects on fertilization membrane, movements of granules and other visible protoplasmic structures, delay in time for first and later cleavages, and irregularities in blastomere formation. Later observations at frequent intervals were sufficient to reveal the ultraviolet-correlated changes in both locomotion and structure that accompanied the successive periods of differentiation of blastulae, gastrulae, and plutei. Each experiment was based on a graded series of ultraviolet exposures, gametes from the same pair of sea urchins being used in each case. A definite injury series was established for both irradiation of unfertilized eggs (subsequently fertilized) and irradiation of fertilized eggs. A direct relationship was noted between dosage and 1) amount of delay in cleavage and later development, 2) percentages of abnormalities produced, and 3) maximal growth attained. Such relationship was readily observed for the more severe doses; it was firmly established also for very mild doses. It was significant that many of the abnormalities resulting from ultraviolet radiation resembled those noted in our parallel x-ray experiments. Illustrative cinephotomicrographs of normal and fast-motion types were obtained which give a vivid portrayal of ultraviolet irradiation effects.

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The extractable proteins of sea urchin embryos. Melvin Spiegel and Evelyn Sclufer Spiegel.

A recent investigation by Kane and Hersh (1959) on the ultracentrifugation of extractable proteins of sea urchin eggs revealed that the bulk of the extractable material was found in two major components. These authors suggested that it was very likely that other soluble proteins were present in the extract but that each must be present in such small concentration that it did not contribute to the observable Schlieren pattern. It was also suggested that the two major peaks themselves are heterogeneous. It was felt that perhaps another method of analysis would reveal whether such heterogeneity exists. Accordingly, 0.1 *M* KCl extracts, pH 7.0, of unfertilized eggs of *Arbacia punctulata* were prepared, adhering as closely as possible to the method of Kane and Hersh. The extracts were then dialyzed in the cold against three changes of Veronal buffer, pH 8.6, $\mu = 0.05$, overnight. The dialysate was centrifuged at 56,000 g for fifteen minutes, the supernate removed by aspiration and subjected to electrophoresis in the Model 38 Perkin-Elmer Tiselius Apparatus at 4° C. and 4 ma. constant current. The results revealed four major components, plus one and possibly two minor components. These relatively simple extracts possessed strong antifertilizin activity when tested against egg jelly, indicating that this procedure may be useful as a starting point in the isolation of egg antifertilizin. Substitution of 0.03 *M* carbonate buffer, pH 10.7, as the extracting medium and using this buffer for electrophoresis at 4° C., 4 ma. constant current, demonstrated eight components. Extracts made of blastulae, gastrulae, and plutei had essentially identical electrophoretic patterns. Attempts are being made to further characterize these components and to relate them to the proteins characterized by ultracentrifugation.

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The magnitude of random threshold fluctuations in the squid giant axon. Charles F. Stevens.

It is known that in the frog node threshold varies randomly around a mean value according to a Gaussian density function with a standard deviation of 1.5–2.0% of the mean, and that there is a variation in latency for a constant stimulus corresponding to this "excitability" fluctuation. In view of these threshold variations in the frog node, it is of interest to investigate the threshold variability of the squid giant axon, in order to determine to what extent any deterministic model may be a fair approximation to the actual case.

A 50 mu axial platinum electrode was inserted into an uncleaned 2½-cm. length of the giant axon from the hindmost stellar nerve of *Loligo pealii,* and constant current pulses were delivered by a modified Grass S4 stimulator which permitted stimulus intensity to be varied reliably by amounts on the order of 1/1500. The sharpness of the threshold for the membrane action potential was determined directly, and also indirectly by measuring latency fluctuations for a constant stimulus. These methods give the sum of fluctuations due to threshold variability and inconstancy of the stimulus.

The principal results are: (1) The threshold determined directly is sharp as far as could be determined by changes of stimulus intensity of 1/1500. (2) There is a latency fluctuation which is largest near threshold, and is, at its maximum, on the order of 10–20 microseconds. (3) A latency variation of this magnitude corresponds to a threshold plus stimulus variation with a standard deviation of 0.015 to 0.020% of the mean.

It is concluded that any random threshold variations in the squid giant axon have a standard deviation less than or equal to 0.02% of the mean. This is considered sufficiently small to justify the application of deterministic models.

Chemically induced hypothermia in the rat: the effect of oxazolium salts compared with 2,4-Dichlorophenoxyacetic acid, ("2,4-D"). F. N. SUDAK, C. LLOYD CLAFF, J. R. BLUMSTEIN AND F. J. TEYAN.

The effects of the oxazolium salt (3-methyl-2(1-naphthyl)-5-phenyloxazolium-*p*-toluenesulfonate) and 2,4-dichlorophenoxyacetic acid on body temperature regulation in male albino rats during cold exposure were compared. Skin and deep colonic temperatures were recorded and carbon dioxide production was measured during a 90-minute exposure to an ambient temperature of 2.0° ± 0.5° C. Saline injected animals, used as controls, showed an increase of 1.5° C. in core-to-skin thermal gradient within 30 minutes after exposure to cold. This thermal gradient was maintained for the next 60 minutes. Colonic temperatures remained unchanged during the entire experiment. In the animals treated with "2,4-D" (300 mg./kg.) and in those treated with oxazolium salt (100 mg./kg.), no significant change in core-to-skin thermal gradient occurred. Colonic temperatures declined in a linear manner at the rate of 0.18° C./min. in both experimental groups.

Carbon dioxide production increased significantly (200–225% above "basal") in the control group within 15 minutes and remained at this level for the remaining 75 minutes. No significant rise (above "basal") in metabolism occurred in the two experimental groups. CO_2 production remained unchanged for 45 minutes and then declined steadily to reach a level 35% below "basal" after 90 minutes in the group treated with the oxazolium compound. Carbon dioxide production declined after 15 minutes in the cold in animals treated with "2,4-D" and reached a level 57% below "basal" at 90 minutes.

Animals treated with the oxazolium salts recovered their normal body temperatures (from 21.0° to 38.0° C.) within 3 hours when placed in an isothermic environment. Recovery of normal body temperature was much slower in animals treated with "2,4-D." Four days after the experiment, 6 of 8 animals treated with the oxazolium salt were dead. No deaths occurred among the "2,4-D"-treated rats for as long as 6–8 weeks (experiment discontinued at this time.)

Muscular activity in albino rats treated with 2,4-Dichlorophenoxyacetic acid, ("2,4-D"). F. N. SUDAK, C. LLOYD CLAFF, F. J. TEYAN AND J. R. BLUMSTEIN.

Muscle potentials of male albino rats treated with 2,4-Dichlorophenoxyacetic acid were studied during lowering of body temperatures under light anesthesia induced with sodium pentobarbital or chloralose. Carbon dioxide production was measured at 15-minute intervals. Rats injected with physiological saline were used as controls. Deep colonic temperatures of the control animals fell sharply during the first 15 minutes and reached a steady state (average of 30.2 ± 1.0° C.) after 60 minutes in an ambient temperature of 12.0 ± 0.5° C.

Recordings of electrical activity in muscles made at each 0.5° C. fall in colonic temperature showed that a sharp rise in activity occurred in the control group at colonic temperatures between 36° C. and 35° C. Muscle potentials continued to rise in amplitude and the frequency of bursts of activity increased as lowering of the body temperature progressed. Muscle potentials reached a maximum amplitude of up to 700 microvolts (average of 7–5 peak-to-peak measurements during high frequency bursts) during the steady-state period. An increase in CO_2 production accompanied the increase in muscle activity and remained constant after 60 minutes in the cold.

In the group of animals treated with "2,4-D," colonic temperatures fell steadily (0.16° C./min.) and a steady-state temperature was never reached during 90 minutes in the cold. CO_2 production did not change significantly for the first 15 minutes and then declined with body temperatures. Electrical activity was not present to any significant degree (122 microvolts recorded in only one animal) at any time in the muscles of animals in this group. The evidence presented indicates that one of the mechanisms of action of "2,4-D" on thermoregulation in the rat is the prevention of shivering.

Thermal gradients in rats treated with 2,4 Dichlorophenoxyacetic acid, ("2,4-D"). F. N. SUDAK, C. LLOYD CLAFF, F. J. TEYAN AND J. R. BLUMSTEIN.

The mechanism of action of 2,4 Dichlorophenoxyacetic acid on physical thermoregulation in rats was investigated. Core-to-skin thermal gradients were measured with thermistors placed 7 cm. into the colon (to a level just caudal to the kidneys in an animal of 150 grams body weight) and on an area of depilated skin just above the first thermistor. Temperature measurements were made on animals in a thermo-neutral environment (28–30° C.) and during a 90-minute exposure to a temperature of $2.0 \pm 0.5°$ C. The average temperature gradient between the core and skin of both control and "2,4-D" treated animals exposed to isothermic temperatures was $1.3 \pm 0.2°$ C. A significant increase (1.3 ± 0.2–2.8 ± 0.4) in the core-to-skin thermal gradient occurred in the control group after 30 minutes in a cold environment and was maintained for the next 60 minutes. Colonic temperatures remained constant during the entire experiment. No significant change in thermal gradient occurred during the 90-minute exposure to cold in the groups of animals treated with "2,4-D." A concomitant decrease in both colonic and skin temperature occurred in a linear manner (0.18° C./min.) in these animals. The evidence presented indicates that 2,4 Dichlorophenoxyacetic acid interferes with those mechanisms which control thermal conductivity in the skin of rats. The possible sites of action of this compound are discussed.

Oxygen consumption in Uca pugnax and pugilator before and after eyestalk removal. F. J. TEYAN, F. N. SUDAK AND C. LLOYD CLAFF.

Oxygen consumption was measured in individual fiddler crabs before and after eyestalk removal. The operations were performed at room temperature. Eyestalkless crabs were divided into two groups: Group I, eyestalks removed and the stubs cauterized with a cautery needle; Group II, eyestalks removed and the stubs left uncauterized. Crabs with their eyestalks intact served as controls. Oxygen consumption was measured every half hour over a four-hour period on four consecutive days, using a modified Scholander method. Oxygen consumption increased within 24 hours after eyestalk removal in the group which was left uncauterized. O_2 consumption either remained the same or decreased over the four-day period in the group in which the stubs of the eyestalks were cauterized. Evidence is presented which indicates that the changes in oxygen consumption in eyestalkless fiddler crabs may be due to the method used in the removal of this organ.

Induced fluorescence in marine eggs. KENYON S. TWEEDELL.

Secondary fluorescence was induced in embryological stages of Arbacia, Chaetopterus, Aeolis and Pectinaria with fluorochromes and studied with ultraviolet light. A high pressure mercury vapor lamp (G. E.) with heat and exciter filters was coupled to a standard monocular microscope having a front surfaced mirror and barrier filter. Several vital fluorochromes, acriflavine, thioflavine and acridine orange were used in concentrations ranging from .001% to .00001% in filtered sea water. Eggs were dyed immediately after shedding (or at various times post fertilization) for 3–5 minutes and then washed in fresh sea water to remove background fluorescence. In the lower concentrations, the eggs developed through the larval stage without being removed from the dye. In either case, the induced fluorescence is retained by the eggs throughout their development. The adaptability of such eggs for cytoplasmic studies is directly related to the amount of yolk platlets, pigment particles, etc. in the cytoplasm. Arbacia was thus unsuitable for study in the early cleavage stages. In other eggs, red, green or gold cytoplasmic granules were traced from early cleavage stages into the larvae. In Chaetopterus, the germinal vesicle and early nuclei appear as darker clear areas surrounded by fluorescing yellow-green yolk platlets. Aeolis fluoresces in various shades of green; thioflavine further differentiates the membrane. Eggs of Pectinaria fluoresce pale green with an orange granular periphery, light green nucleolus in the germinal vesicle, all enclosed in an orange membrane. In all species, spermatozoa appear bright green and their approach and contact with the egg are discernible. Polar body formation and the spindle area in successive cleavages are clearly defined. During the blastula, gastrula and larval stages, the nuclei fluoresce dark green. Fluorochromed embryos which die during development exhibit the Strugger effect: the cytoplasm changes to a brilliant orange and the nuclei fluoresce opaque green.

The effect of bacteria on radiophosphorus uptake of Fucus vesiculosus. WALTON D. B. WATT.

A study was made of the exchange of radiophosphorus between *Fucus vesiculosus* and sea water. The results are expressed as turnover time of plant tissue which is the time required for the total phosphorus in the tissue phase to pass into and out of that phase. Within the limits of the experiments (about 500 ml. of sea water, 2–5 gm. dry weight of *Fucus* and 1 μc. of P^{32}) turnover time was independent of biomass and volume of water. All experiments were conducted in darkness at 23° C. The amount of radiophosphorus (added as phosphate) in the water declined exponentially to an equilibrium value which remained constant for at least 60 hours, an indication that no measurable net uptake of phosphorus occurred during this period. The mean turnover time in freshly collected sea water was found to be 18.8 hours. When the antibiotic tetracycline hydrocloride was dissolved in the sea water (100 mg./l.) the mean turnover time became 37.6 hours. Each value is the mean of four experiments and the difference is highly significant ($p < 0.01$). When autoclaved or millipore-filtered sea water was used, values for turnover time showed close agreement with those obtained using antibiotic. The increase in turnover time with the reduction in bacterial populations was accompanied by an increase in the radiophosphorus accumulated in the algal tissue, suggesting that aquatic bacteria take up the dissolved inorganic phosphorus and release it again in some form (probably organic) which is not immediately available to the plant.

Effects of imposed electrostatic field on rate of locomotion in Ilyanassa. H. MARGUERITE WEBB, FRANK A. BROWN, JR. AND WILLIAM J. BRETT.

Animals were tested by determining the time required to travel a distance of 16 cm. along a section of a plastic tray located directly above a glass-encased 20×40 cm. copper plate. There was a similar copper plate 20 cm. above the test chamber. The copper plates were connected with a constant voltage power supply. In these experiments animals were paired, with one snail being run without electrostatic field for 5 trials and then in an electrostatic field for 5 trials. The other member of the pair was tested for 5 trials with an imposed electrostatic field and then for 5 trials without the field. Voltages used were 350, 500, and 1000. When all of the experiments conducted at 8, 9 and 10 A.M. are considered together it is found that the average test time is reduced significantly when an electrostatic field is imposed. The amount of reduction is 1.7% ($N = 224$, $p < 0.02$, > 0.01). When results obtained in the cases where the experimental situation prevailed in the first 5 trials are considered separately the mean rate is not significantly different from that of the controls. When the experimental condition prevailed in the second 5 trials the mean rate is 3% below the control value ($N = 95$,

$p < 0.001$). In afternoon tests the application of 500 and 1000 volts resulted in a 1.9% *increase* in time required for a trial ($N = 200$, $p < 0.001$). The application of 350 V resulted in a 3.5% *decrease* in time for a trial ($N = 50$, $p < 0.001$). Thus the effect of 350 V is in the same direction as in the morning and is of greater magnitude while the effect of 500 and 1000 V is in the opposite direction and of about the same magnitude.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Fluctuations in rate of locomotion in Ilyanassa. H. MARGUERITE WEBB, FRANK A. BROWN, JR. AND WILLIAM J. BRETT.

The test chamber consisted of a plastic tray divided into 3×16 cm. sections. The tray was located directly above a glass-covered copper plate and 20 cm. below a similar copper plate. The test chamber was illuminated from one end by a lamp with a 60-watt bulb at a distance of 50 cm. For each test the time required for a snail to travel 16 cm. was determined. Snails which failed to cover the distance in 70 seconds in two preliminary trials were not used. Each animal was tested either 5 or 10 times depending on the experiment. Experiments were performed only between the hours of 8 A.M. and 8 P.M., E.S.T., from July 6 to August 14. For each hourly value 20 animals were timed for 5 trials each. A daily rhythm was observed in rate of locomotion with maximum rates occurring at 10 A.M. and 2–4 P.M., and minimal rates at 8 A.M., 12 noon, and 6 P.M. The amplitude of the fluctuations throughout the day represents 20% of the mean daily rate. A comparison of mean daily rates obtained at the same hours of different days showed fluctuations with an amplitude of 29% of the over-all mean daily rate. The rates throughout a series of tests on a single animal were found to vary systematically in a manner characteristic of the time of day. In the morning hours trials 2–5 averaged 1.5 sec. less than the first trial ($N = 100$, $p < .001$) and the reduction was maintained through runs 6 to 10. The mean of rates 2–5 in the afternoon showed a reduction not significantly different from that observed in the morning, but trials 6 to 10 yielded a mean rate not significantly different from 0. These variations in a single test series are of the order of 2 to 3%.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Graded and all-or-none electrically excitable responses of lobster muscle fibers. ROBERT WERMAN AND HARRY GRUNDFEST.

Intracellular depolarization produces only graded responses in stretcher and bender muscle fibers. These arise with vanishingly brief latency to strong stimuli and exhibit refractoriness. Long-lasting depolarizing pulses cause repetitive, damped responses and prolonged strong depolarization produces inactivation. Simultaneous recordings at several intracellular sites demonstrate that the responses propagate decrementally. The maximal amplitudes were 20 to 30 mv, appreciably smaller than the electrically excitable component elicited by stimulating the excitatory axon. This difference may be correlated with the wide distribution along the muscle fiber of excitatory depolarization by the multiterminal nerve supply. The membrane conductance increases about 25% during the response. Replacement of part or all the Na of the bathing fluid with Ba increases the membrane resistance 2- to 10-fold. Stimuli now evoke all-or-none responses of 50 to 100 mv, the amplitude tending to increase with Ba concentration. The responses are prolonged (durations up to 5 seconds), with development of a plateau after the peak. These responses also exhibit properties characteristic of electrical excitability: vanishingly brief latency, marked refractoriness, and inactivation by strong depolarization. Brief hyperpolarizing pulses applied during the plateau can terminate the response. At the peak of the spike, the conductance is increased about 400%, falling slowly during the plateau, and returning to the resting level only after the spike terminates. The impulses are propagated decrementlessly. However, simultaneous recordings at different sites in a muscle fiber reveal different configurations in the responses, which indicate spatial and temporal non-uniformities in the membrane. Unlike insect muscle fibers (Werman and Grundfest, *Fed. Proc., 1959, 18, 169*), Ba-treated lobster muscle fibers do not fire spontaneously.

Mechanism of Ba action on arthropod muscle fibers. ROBERT WERMAN AND HARRY GRUNDFEST.

The conversion of graded, electrically excitable responses of lobster muscle fibers to all-or-none spikes by Ba appears to be due to interference of the ion with K permeability. The resting membrane is increased 2- to 10-fold by low concentrations of Ba with hyperpolarization up to 15 mv. While addition of high potassium to the bathing medium causes depolarization and decreased membrane resistance, simultaneous addition of low concentrations of Ba leads to hyperpolarization and increased membrane resistance. Thus, Ba appears to inhibit K conductance and/or to render the membrane insensitive to K. Loss of membrane rectification, which also results, indicates reduced membrane sensitivity to unequal K distribution, probably caused by diminished K conductance. Since K conductance is probably responsible for repolorization of the action potential, its block by Ba can account for the high amplitudes of the responses, their all-or-none character, and prolonged durations after Ba. Replacement of K by Na, or NH_4 also causes hyperpolarization, although membrane resistance increases only up to 60%. However, all-or-none responses were never seen under these conditions.

In addition to its effect on K conductance, Ba can substitute to a limited extent for Na. After replacement of Na by choline has eliminated responsiveness, application of a 250 mM/L Ba solution frequently restores all-or-none responses. These spikes also are inactivated by depolarization, and, in this condition, brief hyperpolarizing pulses evoke spikes. Thus, the transport and inactivation mechanisms which normally utilize Na during their operation in electrically excitable responses can also utilize Ba. The similar effects of Ba on insect muscle fibers (McCann, Werman and Grundfest, *Biol. Bull., 1958, 115, 356;* Werman and Grundfest, *Fed. Proc., 1959, 18, 169*) suggest that the nature of graded responsiveness in these muscle fibers and the mechanism of Ba action are essentially similar to those in lobster fibers.

Action of x-rays on Paramecium: survival and reproduction. RALPH WICHTERMAN.

Pedigreed clonal cultures of *Paramecium calkinsi* from salt water of West Barnstable and *P. trichium, P. multimicronucleatum* and *P. bursaria* were investigated in regard to the biological effects of x-irradiation on survival and reproductive ability. All species were cultivated in standard lettuce medium with *Aerobacter aerogenes* as the food source. For *P. calkinsi,* the infusion consisted of two parts of lettuce medium and one part of filtered sea water. The irradiation chambers were sealed Nylon syringes of 2 ml. capacity. Usually 200 specimens were placed in each syringe and irradiated, free of air, in steps of 50,000 r up to 450,000 r. After a given dosage, 0.2 ml. of fluid containing a small number of specimens was expressed from a syringe into bacterized spot plates for observation and the establishment of *survival-reproduction* curves. Four syringes were irradiated at one time, each containing a different species. Data were obtained upon 307 isolations in which the fate of 2605 organisms was determined.

Instead of employing the conventional survival-curve in which dosage is plotted against per cent survival, a new type was devised for a minimal 48-hour period which appears to be more expressive of results. Called "survival-reproduction" curves, time in hours is plotted against not only survival of specimens for a given dosage (0–100%) but their subsequent reproductive rates as well. Among other things the data and curves show that *P. trichium* is not only the most radiosensitive but recovery and reproduction occur only at the lower dosages (survival-reproduction rate, 48 hours: control, 278%; 50 kr., 200%; 100 kr., 135%; 200 kr., 28%; 250–400 kr., 0%). On the other hand, *P. calkinsi* is the most radioresistant, showing relatively fast recovery and reproductive ability even after moderately high dosages (survival-reproduction rate, 48 hours: control, 468%; 200 kr., 308%; 250 kr., 267%; 300 kr., 115%; 400 kr., 70%; 450 kr., 18%).

In many instances within the species, the data and curves reveal that with moderately high dosages, reproduction is blocked temporarily for 24–30 hours after which there is not only recovery from irradiation effects but a gradually increased reproductive ability. The greater the dosage, the slower the recovery to reproductive ability.

In nearly all cases, those irradiated specimens which recover to yield large mass cultures have a reproductive rate comparable to the unirradiated controls.

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Responses of the skate, Raja, to hyperglycemic agents. PAUL A. WRIGHT.

The average normal blood sugar value for 20 freshly-caught specimens (probably of mixed species, *ocellata* and *erinacea*), as determined by the Nelson procedure during July and August, was 40.6 mg.% with a great range of variability from a low of 2 to a high of 120 mg.%. Epinephrine (Adrenalin chloride, Parke-Davis), injected intra-arterially at a dosage of 200 mcg./kg., induced a maximal increase in blood sugar of only 14 mg.%, reminiscent of the poor response of bullfrogs to this agent. Cortisone acetate, Merck (1 mg./kg.), did not raise the blood sugar level significantly. Glucose injected into the truncus in a dosage calculated to raise the blood sugar level by 100 mg.% (on the basis of 6% of body weight as the total blood volume) was converted at the following rates: 36% in 5 minutes, 49% in 30 minutes, and 85% in 60 minutes. On the basis of these determinations, the glucose tolerance curve for the skate has markedly different characteristics from that determined previously for the bullfrog. Nine of 10 animals responded to intra-arterial crystalline glucagon with a maximal hyperglycemic rise of 40 mg.% in 60 minutes at a dosage of 100 mcg./kg. and an increase of 68 mg.% in 80–90 minutes at a dosage of 200 mcg./kg. The skate's hyperglycemic reaction to glucagon, then, is comparable to that of the bullfrog but is unlike that of the angler fish (*Lophius piscatorius*), which seemed to be completely refractory to the hormone during the summer of 1958.

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LALOR FELLOWSHIP REPORTS

Functional modifications of sperm structure. MAURICE H. BERNSTEIN.

Electron microscopic investigation of the sperm of *Arbacia punctulata* reveals a wedge-shaped head 1 μ across at the base and 2 μ long. The anterior end has a cup-shaped depression which houses the acrosome granule. The posterior end is indented by the insertion of the tail. The mid-piece is composed of a single ring-shaped mitochondrion with a central hollow, itself doughnut-shaped, containing the centriole and the attachment structures of the tail. The tail has a pair of central filaments with nine paired filaments in radial array forming an outer ring. The central filaments are not present in the proximal half of the mid-piece, but the filaments of the outer ring continue and ultimately participate in the formation of the tail attachment.

Egg-water preparations are known to elicit acrosome reactions in sperm of many marine invertebrates. In egg-water *Arbacia* sperm exhibited a transitory agglutination reaction. Electron microscopy revealed the acrosome still intact, but the mitochondrion of the mid-piece expanded almost explosively.

In nicotin-induced polyspermy release of the acrosome granule to the egg surface was observed. An expansion of the mid-piece comparable to that seen in egg-water preparations was also found. Further direct observations on inseminated eggs have not been made.

In an attempt to reconstruct events following sperm entry, observations were made on sperm suspensions in sea water egg homogenates. An explosive membrane elevation of the sperm nucleus was produced. The tail and the mid-piece seemed very little affected.

The deoxyribonucleoprotein in sea urchin sperm can be solubilized in distilled water after preliminary treatment with a chelating agent and subsequent washing in distilled water. Electron microscopic observations on sperm in the early phases of extraction showed that the head membrane was absent or ruptured, and the nucleoprotein of the head was emerging into the medium.

These and similar experiments aimed at elucidating the modifications of sperm structure and their relations to fertilization are being continued.

Respiration and motility in Spisula spermatozoa. PIERRE H. GONSE.

Experiments have been performed in order to study oxidative metabolism and relate rates of oxygen consumption with motility. Spermatozoa were collected from the punctured gonad. Results described here refer only to material obtained from ripe animals. The spermatozoa were suspended and washed once (by centrifugation at 3000 RPM) in sea water buffered by 0.02 *M* glycylglycine at pH 7.5. Final cell density was from 10 to 30×10^8 cells/ml. Oxygen consumption was measured by polarography; motility estimated by microscopic examination. For endogenous respiration Z O_2 values of 1 were obtained.

"Difference spectra" have been recorded at room or liquid nitrogen temperature in the laboratory of Dr. Britton Chance. In anaerobic suspensions of spermatozoa after treatment by 1 mM KCN, one observes absorption bands corresponding to reduced cytochromes a, a_3, c, and in the region of the b cytochromes, bands at 557 and 562 mμ (low temperature). In the presence of carbon monoxide a cytochrome a_3–CO complex is formed.

The endogenous respiration was augmented 6 to 9 times by 5×10^{-5} *M* dinitrophenol (DNP), a concentration which stops motility completely. A 60% inhibition of respiration occurs in the presence of 5 mM amytal, motility being only slightly lowered. After DNP activation respiration is more sensitive to amytal; a 5 mM concentration produces 90% inhibition. Respiration is unsensitive to 20 mM malonate.

Titrations with azide show two distinct phenomena. The endogenous respiration is stimulated up to 2 times at azide concentrations of 1 to 3 mM, and in this range motility is completely arrested. At higher concentrations this activation and an inhibition are superimposed; different samples yield various results depending upon the degree of activation by 1–3 mM azide. When activation is low, 60% of the respiration is inhibited by 10 mM azide. In the presence of DNP the respiration, which is 8 times greater, is more sensitive to azide; inhibition starts with 0.05 mM azide and is 95% at 10 mM azide. With or without added DNP, the titrations end with the same absolute value of oxygen consumption resistant to azide 10 mM. The inhibition exerted at the level of cytochrome oxidase is therefore a function of the respiratory rate. The activation of respiration at lower azide concentrations may be due to an uncoupling action as in bull spermatozoa.

In *Spisula* spermatozoa motility is more sensitive to uncoupling than to a lowering of the oxygen consumption.

The influence of ribonuclease on the development of the sea urchin. VINCENZO LEONE.

In order to study the proteic organization of sea urchin embryos I have performed experiments that would make understandable the meaning and the importance of ribonucleic acid for morphogenesis of the embryo. I studied the effects of the treatment with ribonuclease on *Arbacia punctulata* (Lam.) embryos, from the unfertilized egg to the early mesenchyme blastula stage. The experiments have been carried out in the following way:

1) Unfertilized eggs were placed in the RNase solution (1‰ in sea water crystalline RNase Sigma) for three hours, and then fertilized; 32% of the embryos reached the pluteus stage, 34% failed to develop entoderm and 34% died.

2) Eggs, immediately after fertilization, were placed in the RNase solution, where they remained for three hours. Twenty per cent of the embryos reached the pluteus stage, 40% failed to develop entoderm and 40% died.

3) A three-hour treatment with RNase was performed on embryos at young blastula stage (that is, 3 to 6 hours after fertilization). The result was: 60% of the embryos reached the pluteus stage, 10% failed to develop entoderm and 30% died.

4) After a 3-hour RNase treatment on swimming blastulae (from the sixth to the ninth hour after the fertilization) 50% of the embryos reached the stage of plutei, 20% failed to develop entoderm and 30% died.

5) Finally, the treatment on early mesenchyme blastula stage (from the ninth to the twelfth hour after fertilization) produced the following result: 50% reached the pluteus stage, 17% failed to develop entoderm and 33% died.

RNase acts by disturbing, in some way, the developmental processes and especially the formation of entoderm. It does not show an animalizing effect; in fact, the ciliary tuft is not increased and the mesenchyme is formed, sometimes in excess. Working with an enzyme that disturbs the embryonic development but does not exhibit animalizing or vegetalizing effect evoked interest in testing the reduction gradients of the embryos by treating them with Janus green 1:200,000. As control I used normal and trypsin-animalized embryos and also SCN-

and iodosobenzoic acid-animalized embryos. The reduction, as it is known, starts earlier in the animalized than in the normal embryos. The reduction activity of the RNase-treated embryos was found to be lower than that of the animalized series and comparable with that of normal embryos.

A rapid method for measuring total lipid, applied to the analysis of the lipids and lipoproteins of Arbacia eggs and embryos. Julian B. Marsh.

Lipids were extracted by the method of Folch, Lees and Sloane Stanley which employs 19 volumes of 2:1 chloroform-methanol followed by mixing with 0.2 volume of water and centrifugation. The washed chloroform extract was taken to dryness in test tubes and heated to boiling with 1 ml. of concentrated H_2SO_4 over an open flame. After cooling, 9 ml. of water were added rapidly and the turbidity measured in a Klett photometer with a green filter. A standard curve with palmitic acid was linear between 0.2 and 1.2 mg. In 34 duplicate analyses, the average error was 3.9% with a standard deviation of 5.3%. Expressed as mg. per mg. of protein, jelly-free Arbacia eggs contained the following: total lipid, 0.50; phospholipid, 0.14; unesterified sterol, 0.041; total sterol, 0.046. No significant changes in these ratios were found 5 minutes after fertilization or after 1, 5, 8, and 22 hours of development. After centrifugation of *M* sucrose homogenates of eggs, the mitochondria were found to contain 38% of the total lipid, 75% of the phospholipid and 44% of the sterol; the microsomes contained 5, 12 and 5% of these fractions, respectively; and the supernatant, 53, 7 and 47%, respectively. By preparative ultracentrifugation of the supernatant of an isotonic KCl homogenate of the eggs, a water-soluble lipoprotein mixture was obtained, having an S_f value of 38.5 at density 1.10. The mixture contained 25% protein, 10% phospholipid, 0.25% sterol and 65% triglyceride. It was separable into 5 components by electrophoresis in 0.1 *M* barbital buffer at pH 8.8.

Inorganic pyrophosphatase and the motility of spermatozoa. Leonard Nelson.

Marine invertebrate and rat sperm contain an inorganic pyrophosphatase distinct from the flagellar adenosinetriphosphatase (ATP-ase). While Mg activates the ATP-ase more than does Ca, the IPP-ase exhibits an "absolute" dependence on Mg; Ca, Mn and Zn permit only negligible activity. Mn in the incubation medium in equimolar concentration with Mg (10^{-3} *M*) causes 75% depression of Mytilus sperm-tail IPP-ase. The relative concentrations of Na-pyrophosphate (NaPP) and Mg may influence, among other parameters, the pH-activity curve, which is bimodal with peaks at neutrality and in the alkaline range. The sperm tails also dephosphorylate Na-tripolyphosphate (NaTPP) and Na-hexametaphosphate (NaHMP) which, together with NaPP, comprise members of a series of polyphosphates. Increasing the concentrations of NaPP and NaTPP above their respective optima causes a sharp drop in enzyme activity. This may not be attributable to an inhibitory effect of accumulated reaction product since the enzyme activity against NaHMP levels off at a maximum in spite of the high inorganic phosphate content of the medium.

Electron micrographs of frozen-dried rat epididymal sperm show increased density of the nine outer longitudinal fibers after incubation in NaPP. After prolonged treatment with 50% glycerol, the fibers lose their reactivity with NaPP, although retaining a positive cytochemical reaction toward ATP. Since pyrophosphate serves as a plasticizer of glycerinated sperm (as well as muscle), these, together with other observations, suggest that control of sperm flagellation may depend on a contraction-relaxation cycle, mediated by the action of NaPP-IPP-ase system regulating the relaxation phase with ATP-ATP-ase dominating the contraction phase, possibly through their mutual requirement for Mg.

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Histochemical studies of the reproductive cycle of Ephelota coronata. Edward E. Palincsar.

The marine suctorian *Ephelota coronata* was used to study factors involved in abnormal reproduction and ageing in a unicellular system. Massive populations were found on *Tubularia* growing along the Woods Hole shore.

The following culture method increased the survival time in the laboratory from 48 hours to 10 days, but with a decreasing reproductive potential. *Campanularia* were inoculated with *Ephelota* and placed in standing sea water which was aerated continuously and filtered through activated carbon. Cultures were maintained at 20° C. at pH 8.0. *Campanularia* were fed daily on *Artemia*, and *Ephelota* were fed two *Uronema* per suctorian per day.

Preliminary histochemical investigations comparing young and adult suctorians were made. The scarlet R method for lipids indicated a 2.4% greater concentration in the adult as compared to young. On feeding there is a great increase in lipid content and the suctorians became opaque. Brachet's method for RNA and DNA showed little difference. Bourne's method showed low xanthine oxidase activity but no relationship to the age of the individual. The xanthydrol technique failed to detect urea. Alizarin Red S method revealed a 3.8% increase of calcium in aged individuals. The Schultz-Smith method and the Gomori method for uric acid gave positive results, but no concentration difference with age. A pigment, tentatively identified as a carotenoid, increased 62.4% with age. Using the mercuric bromphenol technique, a great amount of protein was found in the stalk. Preliminary studies indicate that the rate of synthesis or conversion of the stalk protein is related to the age of the parent individual. Lags of 15–40 minutes were noted in the aged. Preliminary evidence also indicates that the number of buds produced decreases by 20–50% in aged individuals and that the fewer the buds, the larger the total volume of the bud.

Effects of some purine analogs on cleavage in Arbacia punctulata. EDWARD E. PALINCSAR.

Effects of 8-azaguanine and pyrazoloisoguanine on early cleavage of *Arbacia* were examined in order to gain some insight into their action mechanism. The 8-azaguanine was used as the sodium salt. Fertilized eggs were placed in concentrations of the analog in sea water ranging from 5×10^{-5} *M* to 3×10^{-3} *M*. Temperatures were experimentally varied from 20–30° C. At 1×10^{-3} *M*, first cleavage was delayed 30–60 minutes, depending on temperature. Higher concentrations of 8-azaguanine were not used, since it crystallized in sea water. Thirty-minute pretreatments of unfertilized eggs yielded a 20-minute delay in the first cleavage. The effect of 10^{-3} *M* 8-azaguanine on the clear half, the red half and the clear quarter was determined, showing a 20-minute delay in the clear halves and a 15-minute delay in the clear quarters. Five- and 10-minute exposures of zygotes to 10^{-3} *M* 8-azaguanine during the course of first cleavage indicate that greatest inhibition occurs up to late prophase. Ten-minute treatment of unfertilized eggs in 10^{-3} *M* 8-azaguanine showed no change in viscosity, but did show an increase in cortical fluidity.

Using standard methods for the preparation of homogenates from ½ cc. of packed cells, an attempt was made, using spectrophotometric methods and Thunberg techniques, to determine xanthine oxidase activity during early cleavage and in the unfertilized egg. No correlation with 8-azaguanine was obtained and in fact, little to no xanthine oxidase was detected. The techniques may not have been sufficiently sensitive. Pyrazoloisoguanine, both as the hydrochloride salt (1×10^{-4} *M* in sea water), and as a saturated sea water solution, had no effect on cleavage, possibly due to a permeability barrier. These preliminary results suggest that 8-azaguanine action is related to the mitochondrial content of the cell, the period of active protein and nuclei acid synthesis during cleavage and to changes in fluidity of the cortical layer.

On the stability of the structures of animalized and vegetalized sea urchin embryos. SILVIO RANZI.

In previous research, I reached the conclusion that the solution of the proteins of animalized embryos are less stable than the solutions of the proteins of normal embryos, and that the proteins of vegetalized embryos are more stable than the proteins of the normal embryos. The object of the present research was to test this point on the living embryos.

Embryos of *Arbacia punctulata* (Lam.) were animalized by adding 1 ml. 0.5 *M* NaSCN to 9 ml. of egg suspension in sea water, before or after the fertilization. Animalization was also obtained, after fertilization, by 1.3×10^{-3} o-iodosobenzoic acid (IBA) in sea water.

Embryos were vegetalized by adding 1 ml. of 0.5 *M* LiCl to 9 ml. of egg suspension after fertilization. The period of treatment with all these substances was 6 hours. The animalized embryos developed about fifty per cent to plutei and fifty per cent to ciliated blastulae with very long and large ciliary tuft; vegetalized embryos developed about fifty per cent to exogastrulae and fifty per cent to ovoid larvae with a very large intestine.

On these embryos two different series of experiments were carried out.

In the first 2.5 ml. of urea 60 per cent were added to 7.5 ml. of the culture after careful washing in order to remove the previous acting substance. Instead of the urea, 0.2 per cent of trypsin in distilled water can be used (5 ml. trypsin solution to 5 ml. egg suspension). After 15–180 minutes the cultures were washed and put in pure sea water In all these experiments the mortality was very high, the maximum being found in the SCN- or IBA-treated cultures and the minimum in the Li-treated embryos.

The other series of experiments shows that IBA or other animalizing substances, added three hours after the fertilization to a culture of Li-treated embryos, decrease the percentage of vegetalized embryos.

The two kinds of described experiments show that it is possible to observe in the living embryo that the action of lithium is a stabilizing action on the protein structures that opposes demolition by urea, by proteolytic enzymes or by other animalizing substances. On the contrary, the action of animalizing substances lowers resistance to the urea and to proteolytic enzymes.

Further observations relating radiation-induced mitotic delay to centriole damage. RONALD C. RUSTAD.

Two new lines of evidence supporting the hypothesis that radiation-induced mitotic delay arises from damage either to the centriole or a nuclear "trigger" for centriole replication have been obtained.

In *Arbacia* a strong parallelism exists between the pre-fertilization recoveries of x-irradiated eggs from mitotic delay and multipolar spindle induction. Individual eggs dividing multipley after the recovery period are greatly delayed.

Earlier studies of cyclical changes in sensitivity to x-ray-induced mitotic delay were confirmed. Furthermore, the induction of multipolar spindles was found to be dependent on the mitotic stage in the same manner as mitotic delay sensitivity.

Parenthetical to the central problem, numerous observations were conducted on high radiation dose phenomena seen previously in *Lytechinus variegatus.* Tight membranes are sometimes formed. Hemispherical pockets 10 to 30 microns in diameter can appear between the hyaline layer and the cell surface two hours after fertilization. Migration of pigment granules into the furrow region is often enhanced. Micromere formation can be dissociated from the normal cleavage schedule, but not its own "clock," resulting in two- to eight-cell stages containing micromeres. Healthy half-blastulae with degenerating half-morulae appear.

Since the induction of multipolar spindles is accompanied by a retardation of aster multiplication rate and cells with odd-integer numbers of asters occur, the present experiments strongly suggest some form of centriole damage.

This work was performed under the tenure of a Lalor Fellowship.

The inhibition of mitosis in the sea urchin egg by acridine orange. RONALD C. RUSTAD.

Since the diaminoacridines exhibit differential fluorescence when attached to either RNA or DNA *in vivo,* experiments concerning the possible inhibition of mitosis by interference with nucleic acid metabolism in *Arbacia* eggs have been initiated.

Acridine orange (10^{-4} to 10^{-7} M) can block or delay mitosis when administered within 20 to 25 minutes after fertilization. Later addition affects the second rather than the first mitosis.

The dye is actively accumulated by the egg. The higher concentrations stain the jelly coat; removal of the jelly coat does not prevent mitotic inhibition. Condensation of part of the echinochrome within the pigment granules was noted at 10^{-4} to 10^{-5} M concentrations.

Sperm exhibit a time- and concentration-dependent loss of motility and ability to fertilize. Mitosis is delayed when eggs are inseminated with treated sperm. This sperm-induced delay in our present state of knowledge suggests an action on either the nucleus or the centriole.

If the highly selective delay pattern during the division cycle results from action on the same system that causes sperm damage, it would be reasonable to postulate interference with the nucleic acids of the nucleus or the RNA of the centriole. However, the possibility that the cyclical changes might arise from some other effect will require further investigation.

The sensitivity of sperm and the cyclical sensitivity changes of the fertilized egg to acridine orange treatment are very similar to the radiation-induced mitotic delay patterns. Various authors have attributed radiation delay to nuclear damage and the present author has suggested that the radiation effect arises from centriole damage.

This work was performed under the tenure of a Lalor Fellowship.

Neuromuscular mechanisms of sound production in Opsanus tau. C. R. Skoglund.

The functional activity of the intrinsic striated muscles of the swimbladder, which are engaged in sound production of the toadfish (Tower, 1906; Fish, 1954), has been studied by simultaneous recordings of action potentials, mechanical effects, and sound. Most experiments have been performed on the excised bladder kept in air at room temperature (21° C.).

The motor nerve was found to conduct at above 25 m/sec. The shape of the compound action potential indicated fairly uniform fiber sizes, which was confirmed by preliminary histological analysis (fiber diameter 10 μ).

The muscle consists of short fibers running transversely to the long axis of the muscle. A single maximal nerve volley caused a well synchronized contraction as verified by simultaneous recording from different points by steel microelectrodes.

When the muscle contraction was recorded by an RCA transducer with stylus placed against the muscle surface, the earliest detectable movement occurred during the falling phase of the muscle action potential. Contraction peak was reached within 5–8 msec, and relaxation was complete in an additional 5–7 msec. The changes in bladder pressure during contraction, measured by a capacitance manometer, showed a similar time course.

The sound recording with an electrodynamic microphone (60–10,000 cycles) was limited for technical reasons to the initial high-amplitude change. This was recorded as a di- or triphasic wave lasting about 2.5 msec, and audible as a "pop" sound. The first deflection was synchronous with the falling phase of the muscle action potential, and thus, the second occurred during the early phase of recorded muscle movement.

The data are used to interpret the natural sound production of the toadfish, of which some comparative studies have also been made; the results may be relevant to the general problem of muscle sound generation.

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ON THE FOOD OF NUDIBRANCHS

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Apart from certain cases in which still little is known with certainty, our knowledge of the food of nudibranchs is very meagre. Likewise, the question of the food of many of them and its relation to their occasional disappearance by death or migration seem to be matters of confusion among many workers (Hecht, 1895; Chambers, 1934). At Al-Ghardaqa, where it has been possible to observe these animals in their natural habitat and to rear them in laboratory aquaria, advantage was taken to elucidate many points of interest in this regard.

MATERIAL AND METHODS

The species studied were collected from the tidal and subtidal coral patches in the immediate vicinity of the Marine Biological Station at Al-Ghardaqa on the Red Sea (27° 13′ N. Lat., and 34° 45′ E. Long.). The preferred habitat of these animals was found to be on dead coral skeleton overgrown by weeds. It is probable that these animals cannot stand strong light and high temperature for a long time, tending thus to hide by day in crevices. Hence, the best catches were those carried out in the early morning or about dusk. The animals were either killed and examined immediately after collection or reared in laboratory aquaria. The latter had a continuous current of sea water run through fine jets strong enough to form a cloud of air bubbles, thus ensuring perfect aeration and continuous movement of the water. In some aquaria pieces of living stony corals, as for instance *Favia, Goniopora, Coeloria,* as well as many forms of anemones, sponges, and algae, were introduced. Others were deprived of all kinds of food other than the planktonic content of the running sea water. A third group was supplied with sea water which had previously been passed through a cotton filter so as to keep back even planktonic organisms contained in the sea water. The last aquaria had been filled at the beginning of the experiment with filtered sea water so that the animals in them had nothing that they might feed on.

RESULTS

Examination of the gut contents—or the wastes discharged at intervals through the anal aperture—of many of those species kept in the laboratory revealed exceedingly little, as they often contain practically nothing. On the other hand the in-

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vestigation of the gut contents of some chromodorids—killed immediately after collection—does reveal the presence of diatoms and zooxanthellae. In one case of *Chromodoris pulchella,* the oesophagus was found to contain two polychaete post-larvae on their way to ingestion. Careful investigation of its stomach inclusions indicated the presence of small fragments, evidently of the same polychaete species, in various stages of digestion. Included in the gut, also, were a copepod and a considerable amount of calcareous spicules of sponges, as well as very few algal filaments. It seems probable that this individual was feeding on sponges when it ingested some of these polychaete larvae. In many specimens of *Chromodoris quadricolor, Chromodoris annulata* and *Chromodoris ghardaqana,* the gut contents showed nothing but unidentifiable fragments.

Two species of the nudibranch genus *Phyllodesmium* were found to be specific for browsing on *Xenia* and *Heteroxenia* at Al-Ghardaqa (Gohar, 1940; Gohar and Aboul-Ela, 1957b). In sections through their cerata, the latter were found to contain a great amount of zooxanthellae in their hepatic caeca. It would be well to mention here that these zooxanthellae are certainly derived from their alcyonarian food. Thus, when a specimen was taken from a colony of *Heteroxenia fuscescens* which had been kept for more than ten days in a dark room aquarium, it looked as pale as the colony. In sections the cerata were found practically devoid of zooxanthellae.

In another nudibranch—namely, *Dermatobranchus striatus*—the only substratum on which it has been observed is *Clavularia hamra.* Thus, several individuals of this nudibranch were picked out of freshly collected colonies of *Clavularia* immediately after their introduction into laboratory aquaria. The fact that it is always observed on this substratum has led to the conclusion that it is specific for browsing on *Clavularia.* It may be well to refer to the observations of Gohar (1948) in which he points out that *Pleuroleura striata* (a synonym of *Dermatobranchus striatus*) browses on the softer parts of that alcyonarian. In sections, nematocysts were observed in its skin and zooxanthellae in various stages of intracellular and extracellular digestion in its gut. Furthermore, the same author states (p. 22) that, "Both the nematocysts and zooxanthellae are evidently derived from the eaten-up polyps." Although Bergh (1905) gave a detailed description of this nudibranch, no mention was made of its substratum, its food or the possession of nematocysts and zooxanthellae.

By comparing the gut contents of many specimens with the food materials of their habitat, it is found difficult to tell what is actually made use of. To investigate the preferred food taken by some of the nudibranchs under investigation, feeding experiments, using the broth of various animals and plants, were conducted. The most interesting results were those obtained by using sea urchins' gonads, fish, various anemones, crustacean ova, sponges, alcyonarians and algae. Applying the broth of sea urchins' gonads with a pipette to the front end of *Chromodoris quadricolor* or *Chromodoris annulata,* the animal makes progressive movements towards the stimulus and works its oral apparatus continuously, sucking in the broth. When solid pieces of the same food were used, the radula—protruded through the dilated oral tube—is firmly applied to the food material. On retraction, the radular teeth puncture the superficial cells, the fluid contents of which are sucked into the buccal cavity by the muscular contraction and expansion of the buccal mass.

Similar results were obtained by using some algae and crustacean eggs, while the broth of anemones and fish particles—prepared in the same way—were refused.

Other nudibranchs like *Hexabranchus sanguineus* invariably refused all food of animal source but responded well to that of plant origin like pounded algae.

Under field conditions, large colonies of *Discodoris erythracensis,* as well as the tectibranch *Berthellina citrina,* were found to disappear by the end of the breeding season from places where I used to find many of them, together with their spawn ribbons (Gohar and Aboul-Ela, 1957a; 1959). The same took place in the case of *Trevelyana bicolor,* of which twenty-three specimens and twenty-one of its egg-ribbons were collected on June 20, 1952, from a large colony at Qad El-Tair on the shore of Shadwan Island. A month later, a long search to find even a single specimen in this locality proved fruitless. In the laboratory, the animals supplied with most of the food they might have in their natural habitat could be kept in good health for several months. Several of them deposited their egg-ribbons more than twice during the experiment. Although some of the animals supplied with plankton, only, began to show signs of ill-health, others survived quite a long time and deposited their spawn ribbons. The individuals deprived of all kinds of food could hardly survive after the first week. The disappearance of the above colonies should not lead one, therefore, to assume that the animals die after oviposition but—being found accompanied by a large number of their egg-ribbons—it is most probable that they come near shore to spawn and migrate at the end of the breeding season to places where more favourable conditions prevail for the adults.

Discussion

The above observations demonstrate that many of the species investigated show a definite choice of their diet. Thus, while some forms like *Phyllodesmium xeniae* and *Dermatobranchus striatus* subsist mainly on animal matter, which in this case are the alcyonarians, many of the chromodorids here examined subsist for one part of their food on animal matter and for another part on plant diet. A third group like *Hexabranchus sanguineus* is entirely herbivorous, depending more or less completely on vegetable matter for food supply.

The absence of any appreciable amount of food material in the gut of many of the specimens investigated indicates that nudibranchs—as an adaptation to their sluggish life—have acquired the capacity of living on a minimal amount of food. Thus it may be that these animals under normal conditions do not, as a rule, take large amounts of food at one time but browse slowly, and that digestion proceeds as rapidly as ingestion resulting in very little, if any, food material in the guts of even freshly collected specimens.

The relation of food of nudibranchs to their occasional disappearance by death or migration is equally significant. According to one school of naturalists the disappearance of large colonies of nudibranchs can be explained as a result of a reduction in the food supply in a given locality, leading to their death or migration. Another school is of the opinion that this disappearance is primarily due to their immediate death after spawning. In the systematic literature, there is a widespread acceptance of supposed migratory phenomenon succeeding spawning and related processes.

Chambers (1934) recorded the sudden disappearance of *Embletonia fuscata* Gould. two weeks after the collection of several hundred individuals. His efforts to find even one specimen in the same locality were in vain, and this led him to assume the migration of the entire colony. In the opinion of this author such mysterious disappearances and reappearances of colonies at a locality—as recorded in the case of *Embletonia*—can be justifiably attributed to fatal environmental factors such as lack of food, extremes of temperature or the predominance of other life forms predaceous on nudibranchs. According to the same author, Hecht (1895). dealing with *Aeolidia papillosa,* concluded that death follows very shortly after spawning. However, several individuals of *Aeolida papillosa* kept in the laboratory aquaria were reported by Chambers (1934) to deposit their spawn and in no case did death follow oviposition.

In the light of our results, death did occur when the animals were deprived of any supply of suitable food materials, and did not result after reproduction and spawning. The latter processes have successfully been accomplished so long as food, temperature and other environmental conditions are favourable. In no case, whatsoever, did death follow oviposition in our aquaria, but migration may follow spawning in their natural habitat. Our observations, therefore, corroborate Chambers' view and shows that Hecht's conclusion—cited above—may be speculative rather than experimental.

SUMMARY

1. Nudibranchs exercise considerable choice in the selection of their food. There is a complete chain of conditions from animals feeding exclusively on animal diet to animals depending more or less completely on algae for their food supply.

2. Deprived of all other kinds of food, nudibranchs can depend to a certain extent on suitable planktonic organisms for their diet.

3. Death of the animals does not follow reproduction and spawning, but results when the animals lack a suitable supply of food materials.

4. Large colonies of certain species may disappear as a result of migration after spawning to places where more favourable conditions of food, etc., prevail for the adults.

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EFFECTS OF FERTILIZATION AND DEVELOPMENT ON THE OXIDATION OF CARBON MONOXIDE BY EGGS OF STRONGYLOCENTROTUS AND URECHIS AS DETERMINED BY USE OF C^{13} [1]

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In a previous publication, Black, Epstein and Tyler (1958) presented evidence, obtained by the use of a C^{13} label, for the oxidation of CO in fertilized eggs of the gephyrean worm, *Urechis caupo*. In the light the oxidation of CO is superimposed on the ordinary respiration, so that manometrically an excess gas-uptake is observed. The eggs also oxidize CO in the dark, and this masks, in manometric experiments, an inhibition of respiration by this compound.

Studies by other workers showing excess respiration in the presence of CO, as well as oxidation of this compound, were previously discussed (Black *et al.,* 1958; Rothschild and Tyler, 1958). Pertinent experiments, showing such excess gas-uptake in the presence of CO, have been performed on eggs of the sea urchin (Runnström, 1930; Lindahl, 1939; Rothschild, 1949) and the ascidian (Minganti, 1957), on diapausing grasshopper and silkworm embryos (Bodine and Boell, 1934; Wolsky, 1941; Kurland and Schneiderman, 1959), on skeletal and heart muscle of the frog and rat (Fenn and Cobb, 1932a, 1932b; Schmitt and Scott, 1934; Clark, Stannard and Fenn, 1950), on Earle's L-strain cells from the mouse and MK II cells from monkey kidney cultured *in vitro* (Dales and Fisher, 1959), and on leaf tissue of the wild plum (Daly, 1954).

In the early studies on muscle tissue of the frog and rat noted above, the evidence for the oxidation of CO was obtained by manometric methods alone. Similar evidence has been presented by Dales and Fisher for mammalian cells cultured *in vitro,* and by Allen and Root (1957) for whole blood of rats, dogs and men. The use of isotopically labelled CO has provided direct proof of its oxidation in vertebrate muscle tissues (Clark, Stannard and Fenn, 1950) and in whole turtles and mice (Clark *et al.,* 1949). The use of C^{13}-labelled CO also enabled Black *et al.* (1958) to obtain the first direct evidence for its oxidation in marine eggs.

With regard to previous reports of respiratory stimulation by CO in marine eggs, several points of interest may be mentioned. In the sea urchin Lindahl (1939) found a stimulation of respiration of unfertilized eggs by CO both in the light and in the dark. During development, the excess respiration in the light showed a decrease relative to the total respiration. In the dark, the respiration became progressively more inhibited in the presence of CO as development proceeded. A

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similar finding was reported by Minganti (1957) for the eggs of *Phallusia*. These investigators, as well as Runnström (1956), have rejected the possibility of the oxidation of CO in these materials, while Rothschild (1949) considered this to be the most likely mechanism for the excess respiration in the sea urchin.

It was of interest, then, to determine, by use of the C^{13} label, whether or not sea urchin eggs also possess the capacity to oxidize CO and to follow the changes in this property during development in both the sea urchin and *Urechis*.

If one interprets the data of Lindahl (1939) and Minganti (1957) on the premise that CO-oxidation accounts for the excess gas uptake in CO-O_2 mixtures in the light, then their results indicate that during development this capacity decreases relative to the total respiration. The present results with C^{13}-labelled CO show such relative decrease in capacity for CO-oxidation. These experiments also provide data concerning the effect of illumination on the rates of CO-oxidation at various stages in development, and this pertains to the interpretation of CO-inhibition experiments in general.

Material and Methods

Eggs and embryos of the gephyrean worm *Urechis caupo* and the sea urchin *Strongylocentrotus purpuratus* were employed in the experiments. Non-swimming stages were washed by settling in CO_2-free sea water buffered at pH 8.0 with glycylglycine. Top-swimming embryos were collected by filtration through a coarse sintered-glass filter and washed by low-speed centrifugation.

Gas-uptake and CO_2-production were measured with Warburg-Barcroft manometers. In most experiments the vessels employed were of the type designed by Stanley and Tracewell (1955), with two side-arms in the form of hollow stopcocks, which could be closed off from the main chamber, as well as a conventional vented side-arm. In most experiments the vessels contained 3 ml. of egg suspension, 0.3 ml. of 1 N NaOH ("CO_2-free") in the conventional side-arm, 0.4 ml. of 85% phosphoric acid in one stopcock side-arm which was open during the experiment, and 0.3 ml. of "CO_2-free" alkali in the other stopcock side-arm which was closed during the respiration measurement. At the end of an experiment the acid and alkali from the open side-arms were tipped into the main chamber, in order to obtain a measure of the total CO_2 present. After the measurement had been obtained, the closed side-arm containing alkali was opened, allowing the CO_2 to be taken up in the alkali for subsequent analysis of the C^{13}-to-C^{12} ratio. In some experiments conventional vessels were employed, and acid only was tipped in at the end of the run. The alkali was then transferred quantitatively to the Stanley-Tracewell vessels for measurement and recovery of CO_2. In all the instances, retained CO_2 was measured at the beginning of the experiment by tipping acid and alkali into the main chamber of appropriate vessels at the time of the first manometer reading.

The labelled carbon monoxide was prepared from barium carbonate containing 3.85% C^{13}. This was obtained from the Stable Isotopes Division of the Oak Ridge National Laboratories. The method employed was a modification of that described by Bernstein and Taylor (1947), as outlined in a previous paper (Black *et al.*, 1958). In this method the CO_2 generated from the $BaCO_3$ is passed over a zinc dust-asbestos fiber mixture heated to 520° C. in a combustion furnace. The CO formed is mixed with unlabelled CO and stored over 0.1 N NaOH in a gas-bulb attached to a leveling bottle. After storage over alkali for several days, samples of the CO were taken from the vessel and re-oxidized by passing over copper oxide at 400° C. The CO_2 produced was collected in a small volume of alkali and the C^{13}-to-C^{12} ratio was determined. In all cases the expected ratio was obtained, indicating practically complete conversion of the original labelled CO_2 to CO. Duplicate samples taken two weeks apart from one batch of CO indicated that there was no loss of the CO upon storage over the dilute alkali.

After attachment of the Warburg vessels to their manometers the experimental vessels (two in most experiments) were connected to a manifold so that they could be gassed with oxygen simultaneously. Each vessel was flushed with one liter of CO_2-free oxygen from a measuring bottle. The two vessels were then evacuated simultaneously by means of a Toepler pump to $\frac{1}{5}$ atmosphere, and refilled with the labelled CO, as previously described. These procedures required about 30 minutes, and 10 minutes were allowed for equilibration of the vessels in the water bath. Control vessels were left open to the air or gassed with 80% CO–20% O_2 during this time. The experiments were run at 19° C. Shaker speed was 95 c.p.m. at 3 cm. stroke. Illumination was provided by a bank of 30-watt reflector-type G.E. incandescent lamps located below a glass shelf of the water bath. The intensity of the light at the level of the egg suspensions was 1100–1200 foot-candles. For measurement of respiration and CO-oxidation in the dark, one experimental vessel was placed in a lined black bag.

Determination of the C^{13}–C^{12} ratios of the respired CO_2 were made with a Nier mass-spectrometer (Nier, 1947), modified for the detection of small differences in C^{13}–C^{12} ratio (McKinney *et al.*, 1950).[3] In the experiments with eggs of the sea urchins, the CO_2 which was contained in the alkali of the Stanley-Tracewell vessel was diluted with appropriate amounts of a $NaHCO_3$ solution (usually 0.3 ml. of 0.24 M) in order to lower the C^{13}–C^{12} ratio to a range that would be most effectively handled with least danger of "contaminating" the mass-spectrometer.

The alkali and bicarbonate were transferred under CO_2-free atmosphere to reaction vessels wherein the CO_2 was liberated for analysis in the mass-spectrometer. Data obtained in the mass-spectrometer were expressed as deviation of the C^{13}–C^{12} ratio of the sample from that of the standard in parts per mil., *i.e.*,

$$\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 1000.$$

The values obtained in this manner were used in isotope dilution equations for the determination of the amounts of CO_2 which had been derived from the C^{13}-labelled CO.

RESULTS

Manometric data

The rates of gas-uptake and of CO-oxidation in the light and in the dark are presented in Table I for the developmental stages of *Urechis* and in Table II for those of *Strongylocentrotus*.

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[3] The authors are indebted to Mr. Joop Goris for analyzing some of the samples, and to the Department of Geochemistry for the use of the mass-spectrometer.

TABLE I

Rates of respiration and of CO-oxidation in 80% CO/O_2 in light and dark by eggs of Urechis caupo at early and later stages of development

Expt.	Period of development (hours after fertilization)	Gas-uptake ($mm.^3/10^6$ eggs/hr.)			Excess gas-uptake in 80% CO/O_2 ($mm.^3/10^6$ eggs/hr.)		CO oxidized, from determinations of C^{13}/C^{12} by mass-spectrometry ($mm.^3/10^6$ eggs/hr.)	
		In air (light)	In 80% CO/O_2					
			Light	Dark	Light	Dark	Light	Dark
1	1–8	94	150	125	+56	+31	43.2	21.4
2	20–25	663	839	476	+176	−187	105.6	11.7
3	24–30	724	789	395	+65	−329	89.9	4.6
4	24–30	551	593	327	+42	−224	75.8	5.8
5	52–55.5	853	872	384	+19	−469	65.0	6.6
6	52–55.5	792	815	358	+23	−434	60.6	4.8
7	54–57.5	847	883	384	+36	−463	57.5	4.1

In both species, at all stages studied, the measurements show an increase in rate of gas-uptake of the eggs or embryos in 80% CO/O_2 in the light over those in air. In *Urechis* the amount of excess rate of gas-uptake declines somewhat during development, while in *Strongylocentrotus* it increases slightly. However, in both species, the relative excess gas-uptake with respect to the respiration in air is considerably lower in the later embryonic stages than in the unfertilized or freshly fertilized eggs. Thus for *Urechis* the average values of the percentage excess gas-uptake, in 80% CO/O_2 in the light, relative to the respiration in air, are as follows:

TABLE II

Rates of respiration and of CO-oxidation in light and dark by eggs of Strongylocentrotus purpuratus, unfertilized and at early and later stages of development

Expt.	Period of development (hours after fertilization)	Gas-uptake ($mm.^3/10^6$ eggs/hr.)			Excess gas-uptake in 80% CO/O_2 ($mm.^3/10^6$ eggs/hr.)		CO oxidized, from determinations of C^{13}/C^{12} by mass-spectrometry ($mm.^3/10^6$ eggs/hr.)	
		In air (light)	In 80% CO/O_2					
			Light	Dark	Light	Dark	Light	Dark
1	0 (unfertilized)	16.0	29.9	28.5	+13.5	+12.3	8.9	8.0
2	0 (unfertilized)	22.5	40.9	37.5	+18.4	+15.0	13.4	10.6
3	1–5	69.9	97.4	88.3	+27.5	+18.4	20.5	9.9
4	1–7	81.9	118.4	105.0	+36.5	+23.1	24.1	10.8
5	26–30	166.0	190.0	132.0	+24.0	−34.0	25.0	3.4
6	27.5–31.5	200	238	203	+38.0	+3.0	25.0	9.8
7	46–50	211	242	164	+31.0	−47.0	24.4	3.3
8	49–53	264	321	216	+57.0	−46.0	34.0	6.4

	Stage		
	Freshly fert. (1–8 hrs.)	1 day (20–30 hrs.)	2 day (52–57.5 hrs.)
% excess gas-uptake (light)	60	14	3

For *Strongylocentrotus* the average values are:

	Stage			
	Unfertilized	Freshly fert. (1–7 hrs.)	1 day (26–31.5 hrs.)	2-day (46–53 hrs.)
% excess gas-uptake (light)	83	43	17	18

In the dark the eggs of both species show greater gas-uptake in the CO-O_2 atmosphere than in air for the early stages of development and considerable inhibition at the later stages. For *Urechis* the average values of the percentage excess gas-uptake in 80% CO/O_2 in the dark relative to the respiration in air are:

	Stage		
	Freshly fert.	1 day	2 day
% excess gas-uptake (dark)	+33	−39	−55

For *Strongylocentrotus* the average values are:

	Stage			
	Unfert.	Freshly fert.	1 day	2 day
% excess gas-uptake (dark)	+71	+28	−10	−20

These results accord with those of Lindahl (1939) on the sea urchin showing, in CO in the dark, excess gas-uptake for the unfertilized and freshly fertilized eggs,

Table III

Manometrically measured respiratory quotients of eggs and embryos of Urechis and Strongylocentrotus in air and in CO

Experiment	R.Q. in air	Apparent R.Q. in 80% CO/O_2 in the light	Apparent R.Q. in 80% CO/O_2 in the dark
Strongylocentrotus			
1	0.58	0.63	0.73
2	0.85	0.65	0.77
3	0.81	0.77	0.83
4	0.77	0.71	0.85
5	0.85	0.85	0.92
6	0.76	0.77	0.81
7	0.84	0.91	1.0
8	0.81	0.81	1.04
Urechis			
1	0.96	0.98	0.99
2	0.86	1.05	1.0
3	0.82	1.0	1.0
4	0.81	0.92	0.99
5	1.02	0.96	1.08
6	0.81	0.82	1.00
7	0.91	0.92	1.10

and inhibition of respiration for the later embryos. Also, the results obtained in CO in the light accord with those of Lindahl (1939) on sea urchin eggs and Minganti (1957) on ascidian eggs, which showed that the relative excess gas-uptake decreased progressively as development proceeded.

Determinations of CO_2-production were made, as indicated under *Methods,* in these same experiments. Division of these values by the corresponding total gas-uptake gives the respiratory quotients (R.Q.) and apparent respiratory quotients (A.R.Q.) listed in Table III. The A.R.Q. refers to the experiments done in presence of CO since the occurrence of CO-oxidation means that some of the gas-uptake represents CO. Such oxidation has an R.Q. of 2, but manometrically it would be recorded as an R.Q. of 0.67. One would therefore expect that the values for the A.R.Q. in the vessels run in presence of CO would be lower than the R.Q. values in air wherever CO-oxidation occurred.

TABLE IV

Ratio of dark-to-light CO-oxidation

	Rate of CO-oxidation in the dark divided by the rate in the light	
	In *Urechis*	In *Strongylocentrotus*
Unfertilized egg	—	0.79 0.79
Fertilized egg	0.50	0.48 0.45
One-day larvae	0.11 0.05 0.08	0.39 0.13
Two-day larvae	0.10 0.08 0.07	0.18 0.13

The data, however, are mostly in the opposite direction. This would indicate that along with an inhibition of ordinary respiration (in the dark) and its own oxidation, the CO may be inducing a relative stimulation of glycolysis, such as has been reported by Daly (1954) for wild plum, Ducet and Rosenberg (1952) for spinach, Marsh and Goddard (1939) for carrot, Laser (1937) for rat retina and mouse chorion, and Dales and Fisher (1959) for mouse L-strain cells. The data also bear on the question of possible CO_2-assimilation. This is considered in the *Discussion.*

Data from mass spectrometer

The data for the rates of oxidation of CO in the light and dark by the developmental stages investigated are presented in columns 8 and 9 of Tables I and II.

For *Urechis,* the rate in the light is about twice as high in 20–30 hour larvae as in the cleaving eggs. In 50–58 hour larvae, the rate is about one and one-half times that of the fertilized egg.

In the dark, the rate of CO-oxidation in *Urechis* (Table I, column 9) is nearly 4 times greater in cleaving eggs than in the swimming embryos.

For *Strongylocentrotus* the rate of CO-oxidation in the light in fertilized eggs is about twice that of the unfertilized eggs. Following the striking change after fertilization, there is not much increase in CO-oxidation in the light during the remainder of the developmental period investigated. In the dark (column 9) there is no major change in CO-oxidation after fertilization and during later development.

A comparison of CO-oxidation in the dark with that in the light is given in Table IV in terms of the ratio of the two rates at the various developmental stages. In both species this ratio decreases in the later stages. Interpretation of this is offered below on the basis of change in the rate of electron transfer by cytochrome oxidase during development.

For comparison of the rates of CO-oxidation with the excess respiration it should be noted that for each mole of CO_2 derived from CO there would be a manometrically measured gas-uptake of one and one-half moles, according to the equation $CO + \frac{1}{2} O_2 \rightarrow CO_2$. If it is assumed that there is no inhibition of the ordinary respiration by CO in the light, then one would expect the values obtained by mass-spectrometry (column 8 of Tables I and II) for CO-oxidation to equal two thirds of the excess gas-uptake determined manometrically (column 6 of Tables I and II). Such an agreement was found in the previously reported (Black *et al.,* 1958) experiments on freshly fertilized eggs of *Urechis*. It is also found in the present data for *Strongylocentrotus* at all stages and the data for *Urechis* at the early stages. However, for *Urechis* at the 52–57.5 hour stage the excess gas-uptake is considerably lower than expected from the values for CO-oxidation. This evidently means that under the conditions of the experiment, at this stage, there is an inhibition of the ordinary respiration by CO in the light. Possibly this is correlated with the high absolute rate of respiration that the *Urechis* embryos exhibit at this stage. Whether or not increase in illumination would overcome the deficiency in excess gas-uptake (in comparison with CO-oxidation) in *Urechis* at this stage has not as yet been determined.

Discussion

Respiratory inhibition by CO

The mass-spectrometric data for CO-oxidation in the dark permit evaluation of "true" CO-inhibition of ordinary respiration. These values for the inhibition are presented in Table V and are derived simply by subtracting one and one half times the values in column 9 of Tables I and II from the corresponding values in column 5. This is then the respiration in the presence of CO in the dark corrected for CO-oxidation, and its percentage difference from the respiration in air (column 3) gives the inhibition values listed in Table V.

These data show no inhibition of ordinary respiration by CO in the dark for unfertilized and freshly fertilized eggs of *Strongylocentrotus* and for freshly fertilized eggs of *Urechis*. For the later stages of both species there is inhibition of ordinary respiration, the amount of which is, in general, greater the greater the absolute rate of respiration of the air-controls.

A comparison of the data showing lack of inhibition with those of related experiments reported in the literature would be difficult in view of uncertainty, in earlier work, as to the extent to which CO-oxidation occurs in the dark. In any case the lack of inhibitory action of CO in the dark on the early developmental stages does not necessarily imply the absence of a cytochrome system. As Warburg (1927) pointed out, one would not expect a cell to show much inhibition of respiration in the presence of CO unless it was rapidly oxidizing substrate. Thus Runnström (1930, 1932) and Örström (1932) found that when the respiratory rate of *Paracentrotus* eggs was increased by means of dimethylparaphenylene diamine it could be inhibited by CO. Other evidence for the operation of a cytochrome system in eggs of *Urechis* has been previously presented (Rothschild and Tyler, 1958). A brief review is given there, and a more extended one by Runnström (1956) deals with this system in sea urchins.

TABLE V

"True" respiratory inhibition by CO in the dark in developmental stages of Urechis and Strongylocentrotus, obtained after correcting for CO-oxidation

	Percentage inhibition of respiration by CO in the dark	
Period of development	In *Urechis*	In *Strongylocentrotus*
Unfertilized egg	—	0 0
Fertilized egg	0	−5.0 −9.0
One-day larvae	34 46 42	23.5 6.0
Two-day larvae	56 56 55	24.6 21.8

Rothschild (1949) reported an inhibitory effect (Av. 38%) of light on the respiration of unfertilized *Psammechinus* eggs and a smaller effect (Av. 9%) on that of the fertilized eggs. This effect was not found with fertilized eggs of *Urechis* (Rothschild and Tyler, 1958), nor with unfertilized or fertilized eggs of *Phallusia* (Minganti, 1957). It has not, as yet, been investigated with eggs of *Strongylocentrotus*. If such effect occurs with this material, the correction for it would be in the direction that would show an inhibitory action of CO on the respiration of the unfertilized or early stage eggs of this species.

Oxidation of CO

The values given for rates of oxidation of CO in Tables I and II involve the assumption that no CO-fixation occurred and that there was no assimilation of the CO_2 derived from CO in any of the developmental stages. Hultin and Wessel (1952) have found that CO_2-fixation occurs in all stages of the development of the sea urchin pluteus. The extent to which metabolic CO_2 was assimilated in our experiments is not known. If assimilation occurs, then the correction for this would result in larger values for CO-oxidation than those presented here.

From the R.Q. data presented it appears that assimilation cannot account for more than a small fraction of the metabolically produced CO_2. If we assume unity as the expected value of the R.Q. in the absence of assimilation and take 0.85 as a representative value obtained in the present experiments, then there could be

15% CO_2 assimilation. The values for CO-oxidation could then be that much greater than listed.

The participation of Warburg's "iron-containing respiratory ferment" in the oxidation of CO was postulated by Fenn and Cobb (1932b). Subsequently, Breckenridge (1953), using C^{14}-labelled CO and both crude and purified preparations of cytochrome oxidase from heart muscle of the pig, showed that this enzyme is indeed responsible for the oxidation of CO, in the presence of cytochrome *c* and hydroquinone. Evidence somewhat similar to that of Breckenridge is presented in an accompanying paper (Black and Tyler, 1959) for the participation of cytochrome oxidase in CO-oxidation by eggs of the sea urchin.

Breckenridge has based his explanation for the oxidation of CO on the hypothesis that three molecules of cytochrome a and one of a_3 are combined in a single unit, as proposed by Ball *et al.* (1951). According to Breckenridge, if as many as three iron atoms of a tetraheme unit are oxidized and the fourth is reduced and combined with CO, then the CO can be oxidized. On the other hand, if as many as three of the iron atoms are reduced, combination of a_3 with CO will inhibit cytochrome oxidase activity (and presumably the oxidation of CO). He has cited evidence to show that such an inhibition would be expected when the rate of electron-transfer is high (*i.e.,* when cytochrome *c* and reducing substrate are present in excess).

Breckenridge has indicated that under conditions of rapid electron transfer, one might expect a relatively large fraction of the iron to be reduced as compared with the fraction when electron transfer is slow. In the dark, nearly all the reduced iron of a_3 would combine with CO, whereas in the light, even when electron transfer is rapid, only a small fraction of the a_3-iron would be associated with CO, the proportion depending on the intensity of light. Under these conditions, therefore, in the light, CO-oxidation would be accelerated, up to a point, by increasing rates of electron transfer, whereas in the dark, the oxidation of CO would be inhibited when the electron transfer is rapid. The ratio of dark-to-light oxidation would thus decrease with increasing rates of electron transfer. Such a decrease in ratio is found in the developing eggs of *Urechis* and *Strongylocentrotus* as the respiratory rate increases (Table III). If one accepts the above interpretation, it may tentatively be concluded that the rate of electron transfer by the cytochrome system of unfertilized eggs and of early developmental stages is low; *i.e.,* the cytochrome a-a_3 unit is highly "unsaturated" with reducing substrate. This interpretation is consistent with the lack of respiratory inhibition by CO in these stages in the dark. During later development, the cytochrome oxidase molecules become more nearly "saturated" with reducing substrate, leading to more rapid electron transfer, greater respiratory inhibition by CO in the dark, and a lower rate of oxidation of this compound. This interpretation has been used by other authors to explain the differential effects of CO on the respiration of diapausing and non-diapausing silkworm embryos (Kurland and Schneiderman, 1959) and of developing eggs of the sea urchin (Runnström, 1930).

SUMMARY

1. The rates of gas-uptake and oxidation of CO (labelled with C^{13}) have been determined for developing eggs of the gephyrean, *Urechis caupo,* and for unferti-

lized eggs and developing embryos of the sea urchin, *Strongylocentrotus purpuratus*. In the light in 80% CO/20% O_2, there is excess gas-uptake over that of the air-controls at all stages of development, but the percentage of excess uptake falls off as development proceeds. In the dark there is increasing inhibition of respiration by CO during development.

2. The rate of CO-oxidation in the light increases less than two-fold in *Urechis* during 50 hours of development. In *Strongylocentrotus* the rate of CO-oxidation in the light nearly doubles after fertilization, but increases relatively little during later development.

3. In the dark, in *Urechis,* the rate of CO-oxidation during early cleavage is about half that in the light, but the dark-rate decreases five-fold during the swimming stages. In the sea urchin there is only a slight decrease in rate of CO-oxidation during development. The developmental changes in the ratio of dark-to-light oxidation of CO are interpreted on the basis of an increasing degree of saturation of cytochrome oxidase with its reducing substrate.

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CYTOCHROME OXIDASE AND OXIDATION OF CO IN EGGS OF THE SEA URCHIN STRONGYLOCENTROTUS PURPURATUS[1]

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Black and Tyler (1959) have shown that developing eggs of the sea urchin and the gephyrean worm *Urechis* can oxidize CO, and these authors have presented data on the inhibitory effects of CO on respiration of developing embryos. The rates of CO-oxidation and ordinary respiration were such that in the presence of CO an excess gas-uptake occurred in the light in early developmental stages, but the percentage of excess uptake diminished as the respiratory rate increased. In the dark there was increasing inhibition of respiration and, in *Urechis,* diminishing CO-oxidation as the respiratory rate increased.

The inhibitory effect of CO on cytochrome oxidase is well-known. Fenn and Cobb (1932) were the first to suggest that cytochrome oxidase could also oxidize this inhibitor, and Breckenridge (1953) obtained direct proof of this phenomenon, using labelled CO and purified cytochrome oxidase from pig heart. In the latter study, the author suggested a relationship between the relative proportions of oxidized and reduced iron atoms of cytochrome oxidase and the efficiency of CO-oxidation.

In order to interpret the data obtained on CO-oxidation in embryos, it was necessary to determine whether cytochrome oxidase was in the pathway of CO-oxidation in this material, and if so, whether the rates of CO-oxidation in light and dark by enzyme preparations could be changed by altering the amounts of cytochrome *c* in the system. It will be shown that cytochrome oxidase is involved in CO-oxidation in sea urchin eggs, and that the relative rates of oxygen-uptake and CO-oxidation can be altered by changing the concentration of cytochrome *c*.

MATERIALS AND METHODS

Granular preparations of active cytochrome oxidase were made by centrifugation of homogenates of unfertilized, jelly-free eggs of *Strongylocentrotus purpuratus* in 0.1 *M* phosphate, made to pH 7.4 with "CO_2-free" NaOH. The eggs were repeatedly forced through a No. 18 needle into 30 volumes of buffer, and the homogenate was transferred quantitatively to centrifuge tubes. The preparation was centrifuged at approximately 44000 × gravity in an angle head of the Spinco ultracentrifuge for 20 minutes. The sedimented granules were re-suspended in fresh buffer and centrifuged again at the same acceleration. The final sediment was suspended in 0.1 *M* buffer, pH 7.4, to give a concentration of 10%, based on

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original egg volume. Quantitative transfers of homogenates, combined with counts made on aliquots of the original egg suspension, made it possible to base the values obtained for cytochrome oxidase activity and CO-oxidation on egg-numbers, since it could be assumed that a constant percentage of the total activity was recovered in separate runs.

The enzyme activities were measured for one hour in Warburg vessels of the type devised by Stanley and Tracewell (1955), and the CO_2 was measured and recovered for C^{13}/C^{12} analysis. The procedures involved in the measurement of gas-uptake and CO-oxidation have been described (Black and Tyler, 1959). The vessels were illuminated in these experiments by a bank of 150-watt G.E. floodlamps, providing an intensity of 3500 to 5000 foot candles at the level of the enzyme suspensions. In the dark experiment, the measurements were made at night.

TABLE I

Rates of CO-oxidation by granule-preparations in the presence and absence of cytochr me c and ascorbate. Final concentrations, where appropriate, were as follows: egg granul s, 6% (based on egg volumes); cytochrome c, 2 × 10^{-4} M; ascorbate, 0.02 M. All vessels contained PO_4, 0.06 M, pH 7.4, and $AlCl_3$, 8 × 10^{-4} M. Experiments were run in light.

Experiment	Contents of vessel	CO oxidized (mm.^{3}/10^6 eggs/hr.)
1	Ascorbate + cytochrome *c* (blank)	1.3
	Granules only	2.4
	Granules + ascorbate	5.5
	Granules + ascorbate + cytochrome *c*	11.3
2	Granules + cytochrome *c*	3.2
	Granules + ascorbate	4.8
	Granules + ascorbate + cytochrome *c*	20.0

RESULTS

The rates of CO-oxidation by two granule-preparations in the presence and absence of excess ascorbic acid and excess cytochrome *c* are presented in Table I. It can be seen that rates of CO-oxidation are very low in preparations lacking either ascorbate or cytochrome *c*. A low rate of CO-oxidation was also found in the blanks containing ascorbate and cytochrome *c*.

In the presence of both cytochrome *c* and ascorbic acid, rates of CO-oxidation comparable to those of unfertilized eggs and early developmental stages are found. It may be concluded that both these substances are necessary for the efficient oxidation of CO by the granule-preparations.

In Table II, the rates of gas-uptake and CO-oxidation in light and dark are listed for enzyme preparations at three different concentrations of cytochrome *c*. It can be seen that at cytochrome concentrations giving an O_2-uptake of ½ to ¼ the maximum rate, the rate of CO-oxidation in the light is actually higher than that in the vessels containing excess cytochrome *c*. Furthermore, in those vessels in which cytochrome *c* is limiting the rate of O_2-uptake, there is an excess gas-uptake in CO in the light. In the presence of excess cytochrome *c* the O_2-uptake is inhibited by CO in the light. It is of interest that Breckenridge (1953) also obtained excess gas-uptake in CO by his cytochrome oxidase preparations. In the dark the O_2-uptake is strongly inhibited by CO at all three concentrations of cytochrome *c*, but the percentage inhibition is greatest at high concentrations of cytochrome *c*. There is not much difference between the rates of CO-oxidation in the dark at different cytochrome concentrations.

DISCUSSION

It can be concluded from the data of Tables I and II that added cytochrome *c* is necessary for the oxidation of CO by cytochrome oxidase, but the concentration of cytochrome *c* which gives maximum O_2-uptake is higher than the optimum concentration for CO-oxidation in the light.

TABLE II

Rates of gas-uptake and CO-oxidation by granule preparations in the light and dark. All rates are corrected for the autoxidation in the ascorbate-cytochrome c blanks. The vessels contained PO_4, 0.06 M, pH 7.4; $AlCl_3$, 8×10^{-4} M; ascorbate, 0.02 M, and homogenate, 6% (based on egg volumes). Final concentrations of cytochrome c are given below.

Experiment	Cytochrome *c* concentrate in moles/liter	Gas-uptake (mm.^{3}/10^6 eggs/hr.)				CO oxidized (mm.^{3}/10^6 eggs/hr.)	
		Light		Dark		Light	Dark
		Air	80% CO/O_2	Air	80% CO/O_2		
1	2×10^{-4}	762	462			16.3	
	4×10^{-5}	364	382			26.0	
	2×10^{-5}	187	254			21.4	
2	2×10^{-4}	779	495	639	61	16.8	6.1
	4×10^{-5}	356	408	357	139	26.5	6.1
	2×10^{-5}	192	244	217	125	24.0	6.3

The data are of some value in interpreting the changes in CO-oxidation by developing eggs. Black and Tyler (1959) have postulated that in the light, an increasing rate of electron-transfer by the cytochrome system would enhance CO-oxidation up to a point. A comparison of rates of CO-oxidation in the light and no added cytochrome (Table I) with that when there is enough added cytochrome to give ½ maximum O_2-uptake (Table II) shows that the rate of CO-oxidation is increased about 5-fold by this increase in electron transfer. However, at very high rates of activity (excess cytochrome *c*) the rate of CO-oxidation falls. This is in accord with the suggestion of Breckenridge (1953) that CO would inhibit cytochrome oxidase (and presumably CO-oxidation) when a relatively high proportion of the iron of cytochromes *a* and a_3 became reduced, as it might in the presence of excess cytochrome *c* and excess reducing substrate. It can be seen from Table II, column 4 that in the presence of excess cytochrome *c* there is inhibition of O_2-uptake by CO in the light.

The similarity between the excess gas-uptake in CO in the light exhibited by the enzyme preparations at low concentration of cytochrome *c* (Table II), and the gas-uptake in CO by whole eggs during early development is worth noting. For the whole eggs, Black and Tyler regarded the results as an indication that during early development the cytochrome oxidase was "unsaturated" with reducing substrate, as proposed originally by Runnström (1930) on the basis of other experiments. The present finding is in accord with that interpretation.

In the dark, the lack of variation in rate of CO-oxidation at different levels of cytochrome *c* is perhaps not too surprising, when the data are compared with those for intact embryos of the sea urchin, in which the rate of CO-oxidation changes little during development. It should be noted that even at the lowest levels of cytochrome used in the dark, the rate of O_2-uptake by the granules was still much higher than that of early, developing eggs. Presumably if the cytochrome *c* had been reduced still further in these preparations, an excess gas-uptake in CO might have been evident in the dark, as it was in unfertilized and fertilized eggs.

SUMMARY

1. The cytochrome oxidase activity and ability to oxidize CO (labelled with C^{13}) have been determined for sedimented, washed particles obtained from unfertilized eggs of the sea urchin, *Strongylocentrotus purpuratus*. Both added cytochrome *c* and ascorbate were required for the rapid oxidation of CO by the particles, and it is concluded that cytochrome oxidase is in the pathway of oxidation.

2. In the light, the rate of CO-oxidation was found to be greatest at the concentration of cytochrome *c* which gave about ½ the maximum O_2-uptake. When the uptake of oxygen was maximal, the CO inhibited both the O_2-uptake and the CO-oxidation in the light. In the dark the rates of CO-oxidation by the particles were not greatly affected by changes in the level of cytochrome *c*. The results are used as a basis for the interpretation of developmental changes in CO-oxidation in embryos.

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OSMOTIC STUDIES OF AMPHIBIAN EGGS. I. PRELIMINARY SURVEY OF VOLUME CHANGES

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The osmotic properties of eggs of a great many different species have been studied using a wide variety of techniques. The data obtained from such investigations show that on the one hand there are some eggs, such as those of marine invertebrates, which are freely permeable to water and which appear to behave as osmometers when placed in solutions of different osmotic pressures. If the osmotically inactive material within these eggs is taken into consideration, the Van't Hoff-Mariot Law holds over a wide range of osmotic pressures in the form, $P(V - b) = \text{constant}$ in which b is the osmotically inactive material and P and V are the osmotic pressure (or concentration) of the surrounding fluid expressed in appropriate osmotic units, and the volume of the cell, respectively. The review by Lucké and McCutcheon (1932) discusses much of the older literature on the osmotic changes in marine eggs. At the other extreme are eggs of many fresh-water fishes, for example, which appear to be surrounded by a membrane that is completely impermeable to water. Gray (1932) reported that trout eggs are impermeable to both water and intracellular electrolytes, and suggested that it was the vitelline membrane which served as the impermeable barrier. Manery and Irving (1935) showed that when unfertilized or fertilized trout eggs were placed in water their weight increased during the first hour, indicating an uptake of water. But after a short exposure to water there is a change in the eggs and a cytoplasmic membrane becomes impermeable to water. Krogh and Ussing (1937) demonstrated that although D_2O could enter trout eggs when they were first placed in a mixture of D_2O and H_2O, after 6 hours the permeability to D_2O was nil. Fertilized eggs remained impermeable to water during the first 13 days of development.

Eggs of various other species of animals are also laid in fresh water and so are faced with the same osmotic problem as are fish eggs. The eggs of amphibians are in this category. Since amphibian embryology has been studied extensively, particularly the development of frog eggs, it is not surprising to find in the older literature a number of attempts to learn something about the way in which a frog egg avoids destruction resulting from the entrance of large quantities of water at the time it is laid. Unfortunately, there are a number of conflicts in these old data so that even today it is not possible to describe accurately and in detail what happens to the eggs of frogs when they are laid in water. That some sort of change does occur is suggested by the fact that frog Ringer (or 0.66–0.70% NaCl), which is usually considered a suitable medium for ovarian eggs, appears to be hypertonic for fertilized eggs. Standard solution (or 0.38% NaCl) is frequently used as a

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suspension medium for developing frog eggs. Measurements of freezing point depression and of vapor pressure suggest that a fertilized egg actually contains less osmotically active material than an ovarian egg although there is considerable divergence of opinion of the amount of the change and the mechanism responsible for it. Another point of general agreement seems to be that the fertilized frog egg, unlike the egg of the trout, is not surrounded by a membrane impermeable to water. The increase in volume and/or weight which accompanies development is generally interpreted as resulting from a gradual uptake of water.

This early work is reviewed in some detail by Needham (1931) and to a lesser extent by Holtfreter (1943). Very briefly, Backman (1912) and Backman and coworkers (1909a, 1909b, 1912) measured freezing point depressions and concluded that there was a marked drop in the osmotic pressure of the fertilized eggs of *Bufo vulgaris.* This reached a minimum at the early blastopore stage and then rose again, being slightly less than half the value of the ovarian egg before the blastopore closed. They believed that salts did not diffuse from the eggs but rather that the salts became provisionally adsorbed and so were osmotically inactive. On the basis of diameter measurements, Backman showed that unfertilized eggs did not change volume after several hours of exposure to a solution considerably more hypertonic than a solution in which morulae did not change volume. Subsequent workers, including Bialaszewicz (1912) Przylecki (1917) and Krogh *et al.* (1938), could not confirm the tremendous drop in osmotic pressure at the time of fertilization but did find a smaller drop at about this time. The latter group used a vapor pressure technique for measuring total osmotically active materials and in addition made some measurements of total alkali and of chloride. They believed that changes in "total concentration" could be explained on the basis of the entrance of water during early development. They also believed that some salt might be lost from the egg.

Adolph (1927) was one of the few workers who could find no relation between osmotic pressure of the surrounding solution and the volume of frog embryos. He concluded that during the early stages of development the permeability to water and to solutes was somewhat variable, and that the volume of the egg could not be regulated accurately. Holtfreter (1943) studied the effect of distilled water, tap water, standard solution and frog Ringer on the "outline areas" of the eggs of *R. pipiens.* He first studied unfertilized eggs taken fresh from the oviduct and left in their gelatinous capsules. He found that the volume of these eggs decreased markedly when they were placed in frog Ringer "which ought to be isotonic . . ." (pp. 306–307). When these eggs were placed in water they showed a swelling in 24 hours no greater than the swelling in standard solution. Fertilized eggs in distilled water developed normally. He found no significant difference in osmotic behavior between an unfertilized and a fertilized egg "except that the swelling effect of standard solution seems to be more pronounced in the former. It is suggestive to relate the uniformity of the behavior of the fertilized and the unfertilized eggs to the fact, mentioned before, that in any of these media also unfertilized eggs form a vitelline membrane and a solidified surface layer. The latter is, as shall be shown, a most important factor for regulating the internal osmotic conditions" (p. 307). He continues by stating: "The coat in unfertilized eggs is apparently no more permeable than in fertilized eggs. Volumetric changes are therefore a rather unreliable indicator for the actually existing internal osmotic concentration" (p. 308). Fertilized eggs when placed in distilled water shortly after fertilization swell rapidly at first, but following neurulation this rate of swelling decreases. In standard solution the eggs never attain the same initial size as in plain water. In Ringer, these eggs shrink. His data suggest that up to the neurula stage, the osmotic pressure of the eggs is approximately equal to that of standard solution. "The membranes therefore act as an imperfectly regulatory mechanism in establishing an inner milieu which plays the role of an osmotic buffer against the varying outside osmotic pressure" (p. 311). Holtfreter concluded that by the time the eggs were in the oviduct they were isotonic with his standard solution.

The most recent studies of this general nature are summarized by Allende and Orías (1955). This paper reports a rather wide variety of observations concerning the development of frogs that have been made in Houssay's laboratory. The point most pertinent to the present discussion is their suggestion that the change in the resistance to water of the eggs occurs when they are released from the ovary.

The present investigation was undertaken with the hope that it might demonstrate some of the changes which occur in the frog egg before and after it is released from the ovary. The first portion of the work consists of an initial survey of volume changes which occur when ovarian or fertilized eggs are placed in salt solutions of different osmotic pressures. This will then be followed by a more detailed study of the water, sodium and potassium content of eggs. It is thought that perhaps a better understanding of the quantities of these substances in the ovarian, unfertilized and fertilized eggs, and their movements under different conditions may add to an understanding of how these eggs and early embryos "solve their osmotic problems."

Materials and Methods

Since *R. pipiens* does not naturally occur in the vicinity of Bogotá, Colombia, the present studies were made using *Hyla labialis,* the most common local frog, and *Bufo marinus,* a toad which could be readily brought in from the warmer parts of Colombia.

Adult females were selected, the body cavity was exposed by dissection and eggs were removed from the ovary for study. Injections of suspensions of *Bufo* pituitaries in 0.66% NaCl were used to stimulate egg laying and sperm release. Volumes were calculated from diameter measurements made with an ocular micrometer. Since the eggs were essentially spherical, only one diameter was measured and the volume of a sphere with this diameter was calculated. In one experiment the eggs in one solution were not spherical. In this single instance, the largest and smallest diameters were measured and the cells were considered as prolate ellipsoids for volume calculations. In some experiments the eggs with their follicular layers were removed from the ovary and placed directly in approximately 20 cc. of the solution to be studied, in a covered dish. In a few of the experiments, the follicular layers were removed before placing the eggs in the solutions.

RESULTS

Bufo—ovarian eggs

Eggs were removed from the ovary of *Bufo* and no attempt was made to remove the ovarian layers. Twenty-five eggs were placed in each of 5–7 solutions of sodium chloride of concentrations ranging from 0.55–0.75%. Measurements of cell diameters were begun ½–1 hour after the eggs had been placed in these solutions, 1½–2 hours being necessary to complete the measurements. The measurements were then repeated 5–8 hours later (18 hours in one experiment). Only the largest eggs were selected and they yielded sufficiently uniform data so that the diameters of the 25 eggs in one solution were averaged and the volume was calculated from this average value. In order to eliminate the variability in the measurements made on eggs from different females, it was assumed that 0.66% NaCl was isotonic with the eggs and the volume in this solution was given the value of 1.00 The volumes in the other solutions were then divided by the volume in 0.66% NaCl to obtain relative volumes. Typical data are presented in Figure 1. It can be seen that in solutions more hypertonic than 0.66%, there is a shrinking of the eggs, and in more hypotonic solutions, a swelling. In the majority of the experiments, the volumes after several hours' exposure to the solutions were approximately the same as those after the eggs had been in the solutions for approximately an hour.

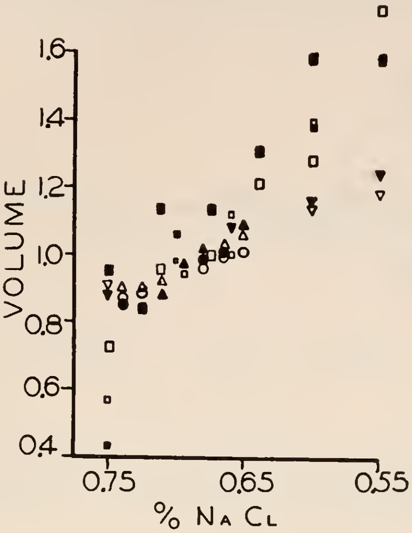

FIGURE 1. Volume changes of ovarian eggs of *Bufo* (with follicular layers) when placed in solutions of NaCl of different concentrations. Open symbols: measurements made ½–2 hours after eggs were placed in the solutions. Closed symbols: measurements made 5–18 hours after eggs were placed in the solutions.

These data were analyzed in the following manner to see if the eggs were behaving as osmometers. The formula $P_1(V_1 - b) = P_2(V_2 - b)$ was used. The relative values were substituted for V_1, V_2, etc. (*i.e.*, each volume was divided by the volume in 0.66% NaCl) and the relative osmotic pressures for P_1, P_2, etc. (*i.e.*, 0.66% was considered to have one unit of osmotic pressure and the other percentages were divided by 0.66 to give relative values of osmotic pressure). In general, these calculations showed that the larger volume changes, such as were measured in the curves indicated by open and closed rectangles in Figure 1, were greater than could be explained on the assumption that the eggs were behaving as simple osmometers (*i.e.*, negative values of b were obtained). The smaller volume changes observed (such as those between 0.75 and 0.65%) would be predicted if the cells were osmometers with a relatively small b-value. Since there is considerable error in the selection of 100 or more eggs all of the same size, which is necessary for such an experiment, and since there is considerable error in the diameter measurements, it is difficult to quantitate such data. However, it seems reasonable to conclude that these eggs (with their surrounding follicular layers) have a tendency to swell as much or more than would be predicted if they were behaving as simple osmometers. There is no evidence that a large fraction of their volumes (b) is reacting as inert to osmotic pressure changes.

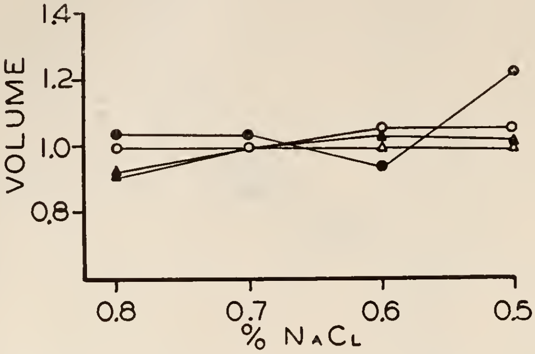

FIGURE 2. Volume changes of ovarian eggs of *Bufo* separated from the follicular layers, when placed in solutions of NaCl of different concentrations. Open symbols: measurements made immediately after eggs were placed in the solutions. Closed symbols: measurements made one hour after eggs were placed in the solutions.

A few measurements were then made using ovarian eggs separated from the follicular layers using jeweler's forceps. Because of the time involved in preparing such eggs, only 6 or 8 eggs were measured and averaged in each solution. The outer layers were removed in 0.66% NaCl, the egg was then transferred to the appropriate solution, the final layer was removed and the diameter was measured immediately. These measurements were repeated at the end of an hour. Measurements made after the eggs had been in the solutions for several hours were not satisfactory due to the distortion of the eggs. The data obtained from two experiments are presented in Figure 2. It can readily be seen that except for one point, the volume changes are considerably smaller than in the preceding experiment (the same scale is used in all of the figures). When these data are treated in the same way as those previously, except for the one large difference between 0.50 and 0.60% NaCl in the one curve, all of the values of *b* are positive and large (60–80%). This is to say, these cells are behaving as simple osmometers with a large portion of their volume being osmotically inactive. Another observation which adds support to this conclusion was the following. When one of these eggs which had become shrunken and distorted in 0.8% NaCl was placed in 0.5% NaCl, it increased in volume and once again became spherical. More experiments are needed to analyze in more detail the difference between the volume changes of ovarian eggs with and without their layers. However, the present data might be interpreted as indicating that the ovarian egg of *Bufo* changed volume very little in solutions of different osmotic pressures, but the follicular layers surrounding it change volume to a much greater extent in these solutions.

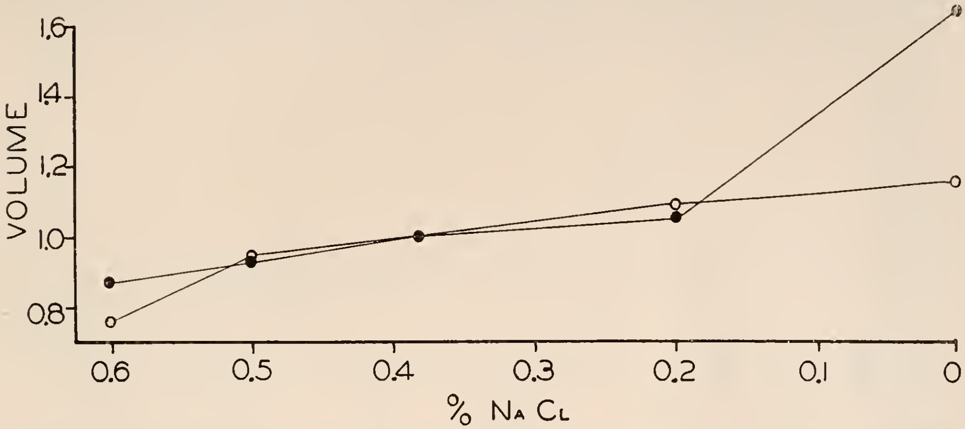

FIGURE 3. Volume changes of fertilized eggs of *Bufo* (with jelly layer) when placed in solutions of NaCl of different concentrations. ○, measurements made after 1½–4 hours; ●, after 24 hours.

Bufo—fertilized eggs

Two experiments were performed using fertilized eggs of *Bufo*. The eggs were laid in tap water and subsequently were separated from the jelly and placed in the appropriate solutions. The layer of jelly immediately surrounding the eggs was not removed. In one experiment the measurements were made between 1½ and 4 hours after placing the eggs in the solutions. The same values were obtained when the measurements were repeated after 8 hours of exposure to the solutions. In the second experiment the measurements were made after the eggs had been in the solutions for 24 hours. In these two experiments the eggs were in blastula or earlier stages. The data are presented in Figure 3. In these experiments, 0.38% NaCl was considered to be isotonic and the calculations of the osmotic behavior were made by assuming 0.38% NaCl is equal to one unit of osmotic pressure and the volume of the eggs in this solution is equal to 1.00. The water used in these experiments was triply distilled from glass, and consequently its osmotic pressure was zero and it was not possible to use these values in the calculations. The majority of the points shown in Figure 3 yielded values of *b* between

60 and 80%. That is to say, the volume changes are in accord with the idea that these eggs are behaving as osmometers with a rather large osmotically inert volume. Similar calculations were also made assuming that 0.60% (in the absence of data in 0.66%) NaCl was isotonic with the eggs. In this case the *b*-values were 90% or larger for the majority of the points. Although this proves nothing, it does suggest that if the eggs are osmometers, more reasonable values of the amount of osmotically inactive material are obtained when a solution with a smaller osmotic pressure is assumed to be be isotonic with them.

Hyla—fertilized eggs with jelly

In this series of experiments the eggs were laid in tap water. They were subsequently removed from the jelly mass, but the layers of jelly immediately surrounding the eggs were not removed, and the eggs were then placed in the appropriate sodium chloride solutions. Diameter measurements were made in some experiments between 1–4 hours after the eggs had been placed in the solutions, in others 3–6 hours and in others 6–8 hours. In most experiments they were again measured after 24 hours. During the early measurements the eggs were in early cleavage or in blastula. The eggs were in gastrula or neurula when the measurements were repeated 24 hours later. Typical data are presented in Figure 4. As in the case of fertilized eggs of *Bufo*, 0.38% NaCl was considered to have an osmotic pressure of 1.00 and the volume of the eggs in this solution was set equal to 1.00. Once again, it was not possible to use the values in water for these calculations, but for all of the other points shown in Figure 4 these eggs were behaving as osmometers with a large percentage of osmotically inactive material.

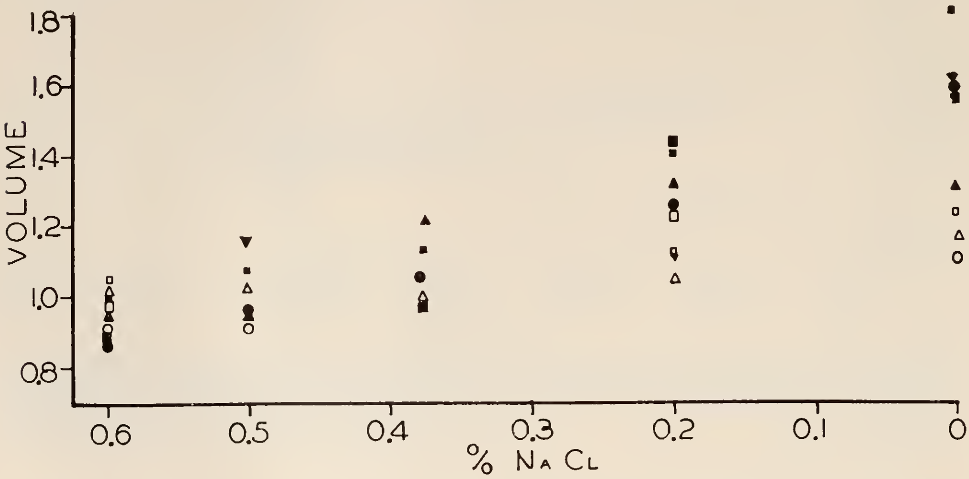

FIGURE 4. Volume changes of fertilized *Hyla* eggs (with jelly layer) when placed in solutions of NaCl of different concentrations. Open symbols: measurements made after 1–6 hours; closed symbols: after 24 hours.

It was generally observed that the rate of development was more rapid in the more hypotonic solutions and also the survival rate was higher (*cf.* Holtfreter, 1943). The data in the next section confirm these observations.

Hyla—fertilized eggs without jelly

In the last series of these preliminary studies, fertilized eggs of *Hyla* with the jelly removed were used. The data obtained from a single experiment are presented in Figure 5. The eggs were measured 20 minutes to an hour and a quarter after having been placed in the solutions, after 18–19 hours and again after 48 hours. The first two series of measurements were essentially the same but after 48 hours the volume changes in the two most hypotonic solutions were much larger than after shorter exposures to these solutions. These eggs, like those with jelly, changed volumes as if they were osmometers with a large percentage of their volume osmotically inactive. At the end of 4 days all of these eggs in 0.6%, in 0.5% and in 0.38% NaCl were dead. In 0.2% NaCl 14% of the eggs were dead after 4 days and 36% after 5 days. After 5 days in water, triply distilled from glass, all of the eggs were living. In another series of observations after 4 days all of the eggs had died in 0.6% NaCl; 64% in 0.5% Nacl, 18% in 0.38% NaCl and all were living in water.

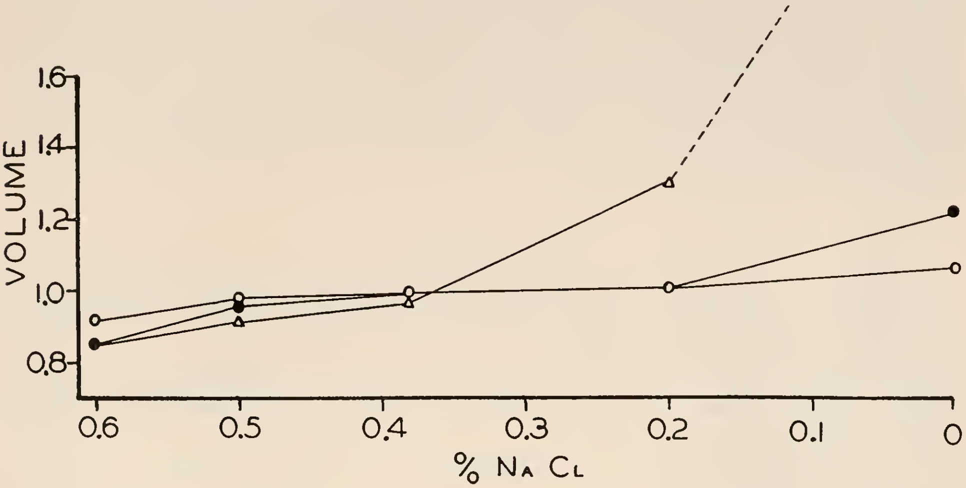

FIGURE 5. Volume changes of fertilized *Hyla* eggs (with jelly removed) when placed in solutions of NaCl of different concentrations. ○, measurements made after ⅓–1¼ hours; ●, after 18–19 hours; △, after 48 hours.

DISCUSSION

The present series of experiments was an initial preliminary survey to determine whether or not the eggs of *Bufo* and *Hyla* would change volumes in solutions of different osmotic pressures. Having shown that this is the case, these changes will subsequently be studied in more detail. Consequently, it is advisable to limit our discussion of the data at this point. However, certain generalities seem fairly obvious. Fertilized eggs of both *Bufo* and *Hyla* change volume in solutions of NaCl of different osmotic pressures in a way that can be reconciled with the theory that they are osmometers with a relatively large osmotically inactive volume. It should be kept in mind that this is not the only explanation possible. The inclusion of the jelly layer in the volume measurements does not appreciably change the "osmotic" behavior of these cells. This might suggest that the jelly layer does not take up or give out large quantities of water in comparison with the eggs themselves. Ovarian eggs separated from the follicular layers behave in a similar manner. That is to say, their volume changes in solutions of NaCl of different osmotic pressures are in accord with the idea that they change volumes as osmometers with a large osmotically inactive volume. When the follicular layers are included in the volume measurements, the changes are much greater. This suggests that the follicular layers take up and give off much more water proportionately than the eggs themselves. In general, all of these types of eggs do not change their volumes much more after 24 hours in a given solution than they do after 2–4 hours. To date, there is no evidence to suggest that during development up to and including neurula stage, there is a difference in the osmotic behavior of these eggs.

Conclusions

1. Ovarian eggs of *Bufo marinus* with follicular layers attached change volume in solutions of NaCl of different concentrations more than would be predicted if they were behaving as osmometers.

2. These same eggs with the follicular layers removed undergo much smaller volume changes. They behave as osmometers with a large percentage of their volumes osmotically inactive.

3. These data suggest that the follicular layers exchange water more readily than do the ovarian eggs.

4. Fertilized eggs of *Bufo marinus* with jelly attached change volume as osmometers with a large percentage of their volumes osmotically inactive.

5. Fertilized eggs of *Hyla labialis* with and without jelly layer also behave as osmometers with a large percentage of their volumes osmotically inactive.

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OSMOTIC STUDIES OF AMPHIBIAN EGGS. II. OVARIAN EGGS

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In the first paper of this series (Luque and Hunter, 1959) it was demonstrated that ovarian and fertilized eggs of *Bufo marinus* and fertilized eggs of *Hyla labialis* undergo volume changes when placed in NaCl solutions of different concentrations. In the present investigation these changes in the ovarian eggs of both *Bufo* and *Hyla* were analyzed in more detail. It is hoped that this type of study may help to elucidate the mechanism of osmoregulation in these eggs and perhaps help to explain the apparent change in the osmotic pressure between the ovarian and fertilized eggs.

Materials and Methods

In order to obtain a better understanding of the volume changes previously observed, the quantity of water, sodium and potassium was first determined on the eggs removed directly from the ovaries of *Bufo* and *Hyla*. The content of these three substances was then determined when these eggs were placed in solutions of NaCl of different concentrations and finally when the eggs were placed in solutions of KCl. The eggs were removed from the ovaries with the follicular layers, and volume measurements were made using an ocular micrometer.

The water content was determined by placing a known number (usually 20–50) of eggs dried on filter paper into small, weighed procelain crucibles. The wet weight was taken immediately and the crucible was then placed in an oven at approximately 100° C. until constant weight was attained. Usually 24 hours were sufficient to reach constant weight. Attempts were also made to determine what per cent of this total water was acting as a solvent. This point seemed of considerable interest because a change in the "binding" of some of the water by cell proteins or in other ways might be an important mechanism in the osmoregulation of these eggs. The distribution method previously used by various authors (see, for example, Parpart and Shull, 1935; Hunter, 1953) was tried, using either glycerol or ethylene glycol. Unfortunately, after many attempts it was finally decided that this method was not satisfactory with these eggs. In every case a quantity of water larger than the total water of the cells was calculated by this method. It is possible that the correction applied for oxidizable material which diffused from the eggs was so large in comparison with the quantity of glycerol or ethylene glycol lost from the solution that the data were unreliable. It is hoped that this problem can be studied in the future using labelled compounds in place of the oxidation-reduction titration.

Sodium and potassium were determined using a Perkin-Elmer flame-photometer with lithium as an internal standard. Triple-glass-distilled water was used in

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[1] The authors are indebted to the Rockefeller Foundation for a grant-in-aid.

every step of these experiments and all of the glassware was cleaned only in bichromate cleaning solution, distilled water and triply distilled water. The remainder of the details of the methods used will be described with a discussion of the results obtained.

RESULTS

Ovarian eggs—Bufo

Before beginning with a study of the changes in Na and K in ovarian eggs placed in different solutions, it was necessary to determine the best method to prepare the eggs before making the salt analyses. The following three methods were tried. (1) The eggs were removed from the ovary and placed directly in a glass tube graduated at 1 cc. The volume was made up to the 1 cc. with triply distilled water and the eggs and water were transferred to a homogenizer. (2) The eggs were separated in 0.66% NaCl, then centrifuged to remove the salt solution, and then placed in the tube graduated to 1 cc. (3) The eggs were separated from the ovary in distilled water, blotted dry on filter paper and then placed in the graduated tube. Fifty eggs were used in every case.

The first method had the possible disadvantage that the ovary might dry out while the eggs were being removed, and so make the eggs abnormal. The second two methods eliminated this problem but added the complication that salts and/or water might exchange between the cells and the bathing solution, or that some of the solution might adhere to the eggs and so change the value of the their salt content.

With each method, once the tube had been filled to the 1 cc. mark with eggs and water, the contents were transferred to a glass homogenizer (a TenBroeck or a Teflon). Ten cc. of triply distilled water containing 154 ppm. of Li were added and the cells were homogenized. One cc. of this homogenate was removed for dry weight determinations (24 hours at 100° C.). The remainder of the homogenate was transferred to a 50-cc. centrifuge tube and 4 cc. of 20% tricloroacetic acid were added. After waiting for at least 15 minutes, the tube was centrifuged (2500 × G) and the supernatant fluid was used for analyses in the flame photometer.

The results of this analysis are shown in Table I. Each value was obtained from a homogenate of 50 ovarian eggs. For example, with toad #2, four separate homogenates were prepared from 200 eggs separated in 0.66% NaCl and 8 homogenates from 400 eggs separated directly from the ovary, 4 separate homogenates from 200 eggs separated in water. The individual data are included in the table to indicate the variability in analyses of different eggs from the same ovary and from different ovaries. The standard errors are included with the averages. The value of *t* was calculated for the difference between the value for sodium of 6.8×10^{-5} meq. per mg. dry weight and the value of potassium of 8.3×10^{-5} meq. per mg. dry weight obtained by method 1. This difference is highly significant and so it can be concluded that these eggs contain more K than Na. The difference between the two values for K obtained by methods 2 and 3 was not significant but the difference between each of these two values (4.5 and 5.7) and the value for K obtained by the first method (8.3) was highly significant. On the basis of this analysis it was concluded that when these eggs were separated in 0.66% NaCl or in water they lost K, and consequently the first method yielded the most reliable data. The Na data did not seem to be appreciably affected by the method. In all of the remaining experiments to be described, the eggs were separated from the ovary of the female and placed directly into the experimental solutions.

TABLE I

The sodium and potassium content of ovarian eggs of Bufo marinus as measured by three different techniques (for details, see text)

Bufo No.	meq./mg. × 10^5					
	Method 1		Method 2		Method 3	
	Na	K	Na	K	Na	K
1	8.4	9.2	5.4	8.6	7.0	7.2
	8.9	9.2	6.1	4.7	8.9	8.9
					8.3	9.5
					8.4	8.4
2	6.9	8.4	7.7	4.1	7.1	3.4
	8.6	8.3	3.7	4.1	6.7	2.9
	5.7	4.5	9.1	2.3	7.2	1.5
	7.5	7.9	9.9	3.4	5.6	1.7
					9.1	2.9
					4.7	2.8
					8.2	1.8
					6.4	2.1
3	7.0	8.5			5.4	7.7
	8.5	8.7			3.2	6.5
	6.8	7.8			6.2	8.9
	9.5	8.5			5.5	7.7
4	8.4	8.8			4.1	6.9
	7.1	9.3			6.3	7.8
	6.8	9.6			5.5	8.2
	5.7	9.4			8.8	7.3
5	6.5	9.6				
	6.6	9.2				
	6.6	8.8				
	4.6	8.4				
	7.5	7.8				
	5.7	6.3				
	4.0	6.7				
	4.5	6.6				
	4.6	7.7				
	8.9	9.3				
Averages	6.8 ± 0.32	8.3 ± 0.25	7.0 ± 0.92	4.5 ± 0.81	6.6 ± 0.36	5.7 ± 0.66

The water content of "normal" ovarian eggs of *Bufo* was determined on three different females. Twenty to 50 eggs with their follicular layers were removed from the ovary, blotted on filter paper and placed in a crucible and weighed immediately and after 24 hours at 100° C. The data are presented in Table II.

Table II

The water content of ovarian eggs of Bufo marinus

Bufo no.	No. of weighings	% water	mg. water/cell
1	17	55 ± 0.4	
2	8	56 ± 1.2	0.58 ± 0.03
3	10	48 ± 1.5	0.70 ± 0.03

As can be seen, not only is there considerable difference between the water content of eggs from different ovaries but also considerable variability with eggs from the same ovary.

Ovarian eggs—Bufo—NaCl solutions

Having determined the values for eggs removed directly from the ovary, the effect of placing the eggs in various NaCl solutions was next studied. Since volume measurements had previously been made (see Figure 1 of preceding paper), they were not repeated in this series of experiments. Eggs were removed from the ovary and placed in the various solutions of NaCl in covered dishes. After two or more hours, 20–30 eggs were removed, blotted on filter paper, weighed and dried for wet and dry weights. Fifty additional eggs were dried on filter paper and placed in the tube calibrated to 1 cc. for the salt determinations. A second homogenate was prepared with another 50 eggs for duplicate determinations. The data obtained from 5 experiments are presented in Tables III and IV. It can be seen from Table III that the changes in per cent water and milligrams of water per egg roughly parallel the changes in volume previously reported. The average relative values are also included (each value divided by the value in 0.66% NaCl). From a comparison between Table I and Table IV it can be seen that in all of the solutions there is a significant increase in the Na content of the eggs but the amount of K does not change appreciably.

Table III

Changes in water content of ovarian eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	Control		0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	—	—	72	1.34	63	0.90	65	1.09	68	1.09	77	1.83
2	—	—	64	1.28	66	1.61	66	1.55	65	1.43	71	2.12
3	49	0.60	45	0.61	57	1.02	58	1.05	58	1.01	65	1.41
4	51	0.72	56	1.09	53	0.83	59	1.04	64	1.20	60	1.00
5	43	0.78	55	1.04	49	0.90	56	1.12	54	0.98	59	1.25
Average	48	0.70	58	1.07	57	1.05	61	1.17	62	1.14	66	1.52
Average (relative)			0.95	0.91	0.93	0.90	1.00	1.00	1.02	0.97	1.08	1.30

Table IV

Changes in salt content (meq./mg. × 10^5) of ovarian eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	14.5	6.3	17.4	7.6	15.0	8.2	14.8	9.2	14.2	9.1
2	16.6	8.8	15.5	7.8	15.8	9.1	16.3	8.6	13.0	7.6
3	13.0	8.5	12.5	7.4	11.2	6.4	12.9	9.8	7.4	6.1
4	13.0	8.0	12.8	7.7	12.0	8.1	16.8	8.4	11.3	7.8
5	7.1	6.9	6.9	6.5	7.5	7.2	6.9	6.4	6.5	7.6
Average	12.9	7.7	13.1	7.4	12.2	7.8	13.5	8.4	10.5	7.6

Ovarian eggs—Bufo—KCl solutions

A similar series of observations were then made, placing ovarian eggs of *Bufo* in solutions of KCl. As before, the eggs were removed from the ovary and placed in solutions of KCl in covered dishes. After two or more hours, the diameters of 10 eggs were measured, 20 eggs were selected for weighing and 50 eggs for salt determinations. Figure 1 shows that the volume changes were small with *b*-values positive ranging from 0 to about 0.80 (the same scale has been used with these figures as was used in the preceding paper). The data for water content (Table V) show changes similar to the volume changes. Table VI shows that in all of the solutions, Na leaves the cells and K enters.

Ovarian eggs—Hyla

Measurements were made using ovarian eggs of *Hyla* (with follicular layers attached) from three females, to determine the "normal" content of water, Na and K in these eggs. Three or four groups of 25 eggs were placed in crucibles

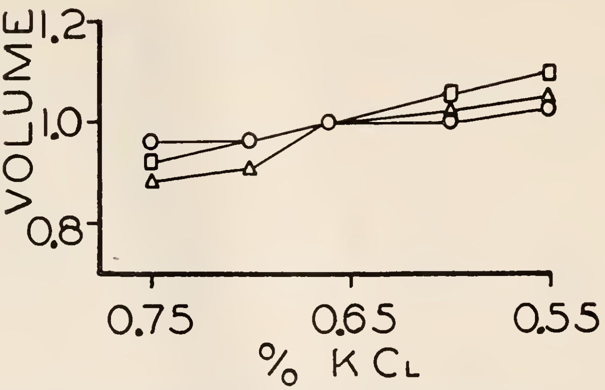

Figure 1. Volume changes of ovarian eggs of *Bufo* (with follicular layers) when placed in solutions of KCl of different concentrations. Measurements made after two or more hours.

Table V

Changes in water content of ovarian eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	Control		0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	50	0.50	52	0.77	61	0.79	61	0.87	62	0.81	64	0.91
2	48	0.57	66	1.15	70	1.21	70	1.31	72	1.42	74	1.48
3	41	0.35	47	0.40	63	0.63	61	0.61	62	0.71	65	0.83
Average	46	0.47	55	0.77	65	0.88	64	0.93	65	0.98	68	1.07
Average (relative)			0.86	0.83	1.02	0.95	1.00	1.00	1.02	1.05	1.06	1.15

for wet and dry weight determinations and three or four groups of 50 eggs were used for salt determinations. The values for per cent of water, quantity of water per egg and milliequivalents of Na and K per milligram dry weight are shown in Table VII. These eggs contain slightly more water than those of *Bufo* but less sodium and potassium. There is less difference in these *Hyla* eggs between the amount of Na and K than in the *Bufo* eggs but there is significantly more K in both types of eggs.

Ovarian eggs—Hyla—NaCl solutions

Similar observations were then made using *Hyla* ovarian eggs in solutions of NaCl. Because of the smaller number of eggs in each ovary, the 10 eggs used for diameter measurements were subsequently added to 40 other eggs for salt determinations and only one homogenate was prepared. Twenty eggs were used for wet and dry weights. These data are presented in Figure 2 and in Tables VIII and IX. All of the changes observed are very similar to those obtained with *Bufo*. The volume measurements were made after one or two hours' exposure to the solutions and the observed changes (Fig. 2) are small. The largest changes are slightly greater than would be predicted if these eggs were osmometers

Table VI

Changes in salt content (meq./mg. $\times 10^5$) of ovarian eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	4.5	11.8	3.0	10.8	3.8	9.2	2.6	10.6	3.2	8.8
2	6.8	15.1	4.7	12.3	1.5	11.7	7.0	9.1	9.2	9.0
3	3.1	8.1	3.3	20.1	2.4	17.7	2.0	14.7	1.2	15.2
Average	4.8	11.7	3.7	14.4	2.6	12.9	3.9	11.5	4.5	11.0

TABLE VII

The content of water, Na and K in ovarian eggs of Hyla labialis

Hyla no.	No. of weights	% water	mg. water/egg	meq./mg. × 10^5	
				Na	K
1	3	64 ± 0.57	1.02 ± 0.039	4.3 5.0 5.5	4.6 5.5 5.5
2	4	49 ± 0.83	0.72 ± 0.029	3.6 3.8 3.6 3.6	5.4 5.7 5.2 5.2
3	4	52 ± 0.50	0.65 ± 0.024	5.1 5.1 4.5 4.8	5.6 5.6 5.4 5.3
Averages		55	0.80	4.4 ± 0.20	5.4 ± 0.028

(negative *b*-values) but the majority of the differences yield positive values of *b*. The changes in water content (Table VIII) roughly parallel the volume changes. These cells gain a considerable quantity of Na and lose a small amount of K (Table IX).

Ovarian eggs—Hyla—KCl solutions

The last series of measurements were made using ovarian eggs of *Hyla* in solutions of KCl after a one-hour exposure to these solutions. These data are

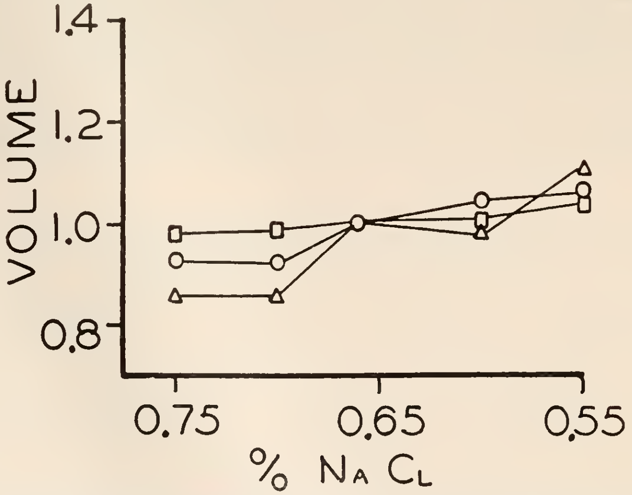

FIGURE 2. Volume changes of ovarian eggs of *Hyla* (with follicular layers) when placed in solutions of NaCl of different concentrations. Measurements made after one or two hours.

TABLE VIII

Changes in water content of ovarian eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	Control		0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	47	0.46	45	0.39	48	0.51	48	0.52	48	0.49	48	0.46
2	64	0.80	56	0.50	56	0.62	61	0.64	60	0.63	61	0.64
3	50	0.66	45	0.55	47	0.60	51	0.76	50	0.72	59	1.03
Average	54	0.64	49	0.48	50	0.58	53	0.64	53	0.61	56	0.71
Average (relative)			0.92	0.75	0.94	0.91	1.00	1.00	1.00	0.96	1.06	1.11

presented in Figure 3 and in Tables X and XI. As before, the largest volume changes give negative values of b but the majority of changes could be explained on the basis of the cells being osmometers. Once again, the changes in water content are similar to the volume changes (Table X). The quantity of Na in these eggs does not change appreciably but there is a large increase in the amount of K (Table XI).

DISCUSSION

The errors involved in a study of this type make it difficult to draw strictly quantitative conclusions. For example, from one toad or from one frog to another, particularly when using species which are not exposed to marked seasonal changes, the size of the largest eggs in the ovary will vary considerably. The eggs which showed the largest volume changes in solutions of NaCl, in the study of *Bufo*, had an average volume of 0.542 mm.^{3} in 0.66% NaCl while the eggs which showed much smaller volume changes had an average volume of 2.580 mm.^{3}. If the conclusion is correct that the follicular layers change volume proportionally much more than the eggs (*cf.* Luque and Hunter, 1959), this might explain the larger volume changes with the smaller eggs. But since it is obviously impossible

TABLE IX

Changes in salt content (meq./mg. × 10^5) of ovarian eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	0.75% NaCl		0.70% NaCl		0.66% NaCl		0.60% NaCl		0.55% NaCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	4.8	3.3	6.1	3.0	5.0	3.4	7.0	3.5	6.3	2.9
2	9.2	3.6	8.4	3.9	7.2	3.7	8.6	3.4	7.1	3.8
3	11.0	4.7	10.0	3.0	9.3	2.3	9.2	2.3	13.0	2.8
Average	8.3	3.9	8.2	3.3	7.2	3.1	8.3	3.1	8.8	3.2

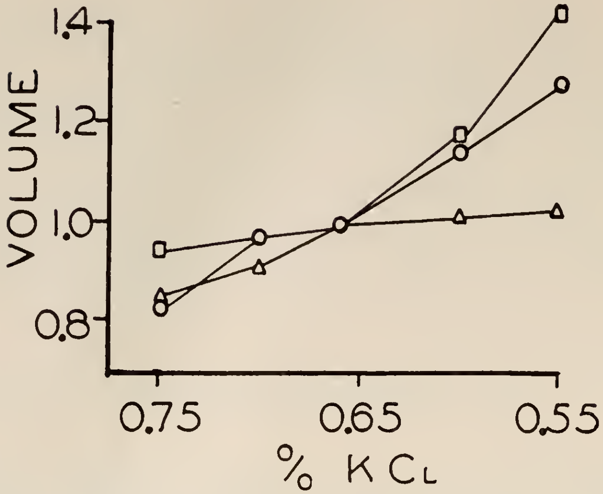

FIGURE 3. Volume changes of ovarian eggs of *Hyla* (with follicular layers) when placed in solutions of KCl of different concentrations. Measurements made after one hour.

to study all of the aspects of a problem such as this at one time, it seems best to use the material, heterogeneous as it may be, and see what general information can first be obtained.

Ovarian eggs of *Bufo* (with follicular layers) have a water content of 50 ± 10%, 6.8×10^{-5} milliequivalents of Na per mg. dry weight and 8.3×10^{-5} milliequivalents of K per mg. dry weight. From Table II, using the data from *Bufo* 2 and 3 we obtain the values of 52% water and 0.64 mg. of water per egg. The dry weight of each egg, then, is 0.59 mg. Multiplying this weight by the values for Na and K we see that each egg contains 4.0×10^{-5} milliequivalents of Na and 4.9×10^{-5} milliequivalents of K. Knowing the quantity of water in each egg we can calculate the sum of the milliequivalents of these two cations in one liter of egg water. With the above data, this has the value of 140. That is to say, con-

TABLE X

Changes in water content of ovarian eggs of Hyla labialis placed in solutions of KCl of different osmotic pressures

Hyla no.	Control		0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	48	0.61	60	1.01	60	1.03	60	1.04	60	1.06	63	1.26
2	48	0.69	69	1.47	70	1.84	70	1.68	73	1.51	73	1.53
3	58	0.85	70	1.43	73	1.67	73	1.68	73	1.83	74	1.86
Average	51	0.72	66	1.30	68	1.51	68	1.47	69	1.47	70	1.55
Average (relative)			0.97	0.88	1.00	1.03	1.00	1.00	1.02	1.00	1.03	1.06

TABLE XI

Changes in salt content (meq./mg. × 10^5) of ovarian eggs of Hyla labialis placed in solutions of KCl of different osmotic pressures

Hyla no.	0.75% KCl		0.70% KCl		0.66% KCl		0.60% KCl		0.55% KCl	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	3.2	11.4	3.7	13.2	5.6	11.8	3.5	11.5	3.2	12.9
2	5.6	19.0	4.6	14.5	7.9	19.4	3.7	18.0	5.9	17.0
3	4.0	17.4	3.1	17.4	3.9	16.0	2.5	14.8	2.5	15.2
Average	4.3	15.9	3.8	15.0	5.8	15.7	3.2	14.8	3.9	15.0

sidering only Na and K, these eggs have a salt content equivalent to 0.140 *M* NaCl. This value seems a little high but at least it suggests that two assumptions are probably quite reasonable: (1) that Na and K constitute the major quantity of cations in these eggs and (2) 0.66% NaCl should have an osmotic pressure much closer to that of the eggs than 0.38% NaCl.

When *Bufo* eggs are placed in solutions of NaCl with osmotic pressures not much different from that which is assumed to be isotonic, the per cent of water and the amount of Na in the eggs increase in all of the solutions, but the amount of K does not change appreciably. In solutions of KCl of the same concentrations, the per cent of water increases approximately the same amount, the amount of K increases and Na decreases. These observations show that water, Na and K can move into and out of the eggs to a limited extent, at least. The cations did not exchange to diffusion equilibrium during the duration of these experiments. The question as to whether or not these *Bufo* eggs are behaving as osmometers is diffi-

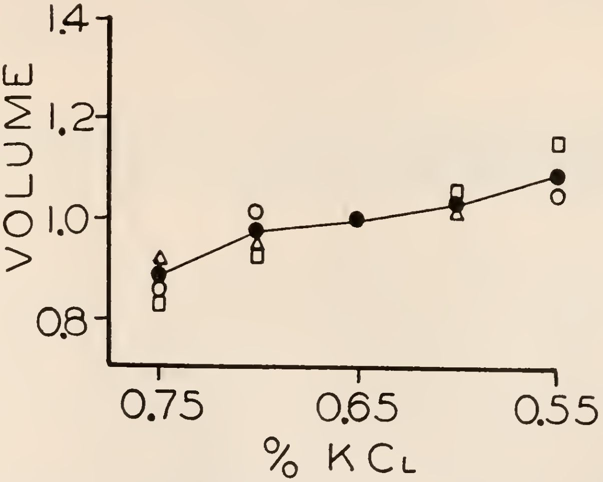

FIGURE 4. Ovarian eggs of *Bufo* (with follicular layers) when placed in solutions of KCl of different concentrations. △, volume changes; ☐, water content per egg; ○, % water; ●, average of the three preceding values.

cult to answer because of the variability of the data and the errors involved in the measurements. In general, however, the majority of the data are not inconsistent with the hypothesis that these *Bufo* eggs are changing volume as osmometers with a relatively large proportion of their volumes osmotically inactive. Since the dry weight of these eggs is equal to 50 ± 10% of the volume, the *b*-value would have to be approximately 0.5 if all of the cell water were osmotically active. Three different values have been used to try to measure volume changes: (1) diameter measurements; (2) % water; and (3) quantity of water per egg. The last two values depend on wet and dry weights but the amount of water per cell takes into account differences in the size of the eggs. In general, when these three sets of data are calculated as relative changes (considering the value in 0.66% NaCl as 1.00) they are very similar. In Figure 4 are plotted these data for the eggs of *Bufo* in KCl solutions. The line connects the averages of these three values. The four points for 0.70% to 0.55% NaCl essentially form a straight line with a *b*-value of 0.52. This would suggest that in these four solutions, at least, these cells were behaving as osmometers with all of the cell water being osmotically active.

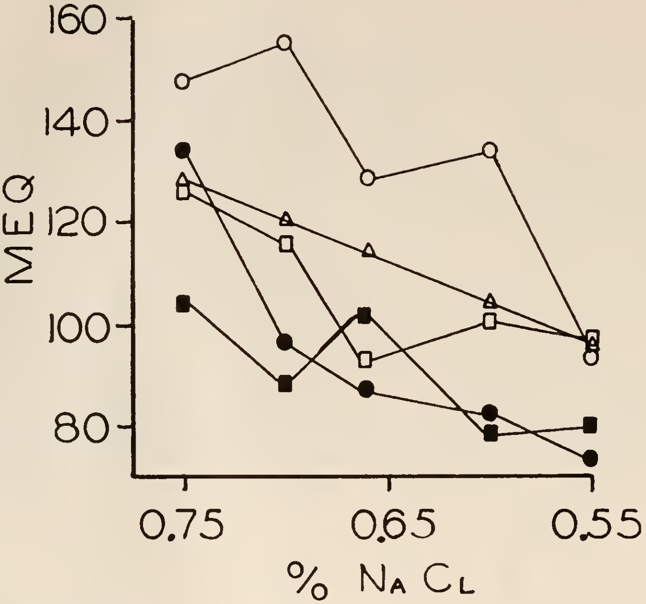

FIGURE 5. Milliequivalents of cations per liter of ovarian egg water in solutions of different salt concentrations. ○, eggs of *Bufo* in solutions of NaCl; ●, eggs of *Bufo* in solutions of KCl; □, eggs of *Hyla* in solutions of NaCl; ■, eggs of *Hyla* in solutions of KCl; △, calculated values (% NaCl converted to milliequivalents).

Another way of analyzing the data is shown in Figures 5 and 6. Using the method previously described, the number of milliequivalents of Na plus K in a liter of cell water was calculated from the values in Tables III, IV, V and VI (Tables VIII, IX, X and XI for *Hyla*). The straight line (Fig. 5) merely represents the milliequivalents of Na in each of the 5 solutions. If the quantity of water and/or cations is changing as a consequence of the change in the amount of cation in the surrounding solution (*i.e.*, osmotic pressure), one would expect that these eggs would have a smaller concentration of cations when placed in the less concentrated salt solutions. This might result either from the entrance of water or the exit of cations. If the eggs were behaving as osmometers, the curve relating the concentration of cations within the eggs with the external salt concentration would be parallel with the theoretical curve. On the other hand, if the eggs did not gain or lose water and/or cations, these experimental curves should be parallel with the x-axis. Although the curves in Figure 5 are far from smooth, their slopes much more nearly parallel the theoretical straight line than the x-axis. Figure 6 shows that it is the quantity of water that is changing and not the total quantity of cations or of Na or K. These curves in Figure 6 are roughly parallel with the x-axis. The curves with the open symbols show the sum of Na plus K in solutions of NaCl and KCl while the curves with the closed symbols show the Na content in these two sets of solutions. Considering the *Bufo* eggs, the sum of Na and K, as well as their concentrations, is higher in the NaCl solutions than in KCl. This might result from the apparent difference in the net exchange of Na and K. In solutions of NaCl more sodium appears to enter the cells than K enters in

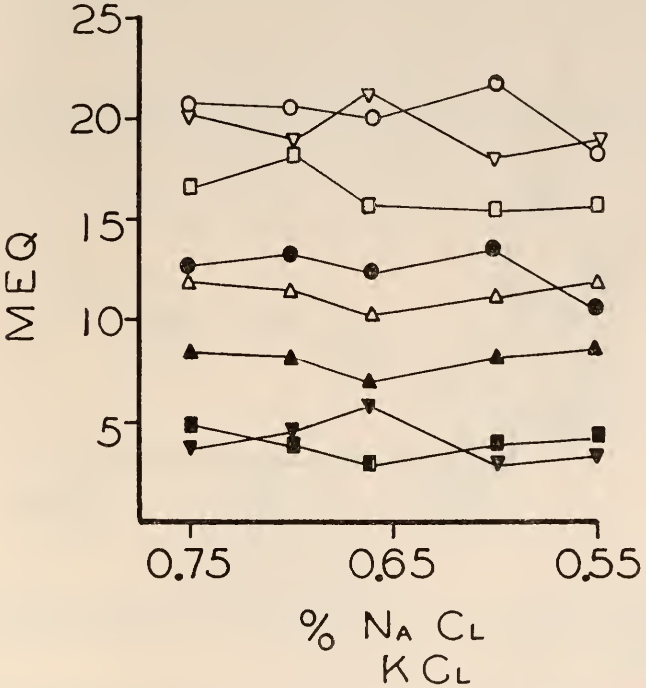

FIGURE 6. Milliequilivants of cations (× 10^5) per ovarian egg in solutions of different salt concentrations. ○, sum of Na and K—*Bufo* eggs in NaCl; ●, Na—*Bufo* eggs in NaCl; ▽, sum of Na and K—*Hyla* eggs in KCl; ▼, Na—*Hyla* eggs in KCl; □, sum of Na and K—*Bufo* eggs in KCl; ■, Na—*Bufo* eggs in KCl; △, sum of Na and K—*Hyla* eggs in NaCl; ▲, Na—*Hyla* eggs in NaCl.

solutions of KCl. Furthermore, more Na leaves the cells in solutions of KCl than K leaves the cells in solutions of NaCl. As a consequence of this apparent more ready exchange of Na than K, the eggs in NaCl solutions contain more cations. The salt osmotic pressure in the eggs in solutions of NaCl appears to be greater than that of the surrounding solutions, whereas in KCl solutions, it is less. Whether this is a rate or equilibrium difference cannot be determined by these studies.

Turning now to a similar analysis of the data obtained with *Hyla* ovarian eggs, their water content seems to be slightly higher than that of *Bufo* (55 ± 10%), and the Na and K contents slightly less (Na: 4.4 × 10^{-5} milliequivalents per mg. dry weight; K: 5.4 × 10^{-5} milliequivalents per mg. dry weight). Both types of eggs have slightly more K than Na. Without going through the details a second time, the data on volume measurements, per cent water, and water content per egg are not inconsistent with the idea that these eggs also behave as osmometers, within the limited range of solutions used, with all of the water being osmotically active. Figure 7 compares with Figure 4. The data for *Hyla* are also included in Figures 5 and 6. From Figure 5 it can be seen that in solutions of NaCl the cation concentrations within the eggs are essentially equal to the concentration in the surrounding solutions. This would suggest that the osmotic pressure of *Hyla* eggs is somewhat less than that of *Bufo* eggs. Once again, the concentrations of the cations are less in the KCl solutions than in the NaCl solutions but the quantity of cations was greater in the cells in the KCl solutions, resulting from the rather large amount of K which entered into the eggs.

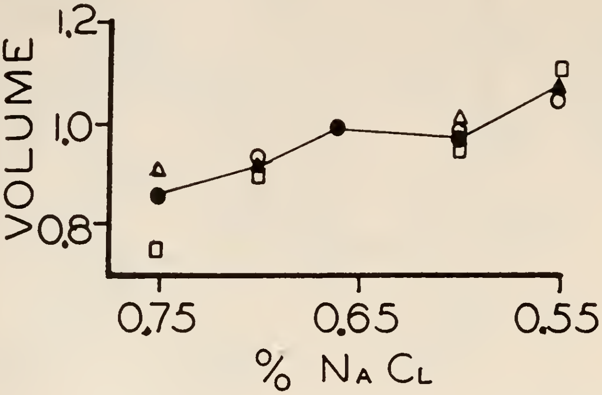

FIGURE 7. Ovarian eggs of *Hyla* (with follicular layers) when placed in solutions of NaCl of different concentrations. △, volume changes; □, water content per egg; ○, % water; ●, average of the three preceding values.

CONCLUSIONS

1. Ovarian eggs of *Bufo marinus* (with follicular layers attached) contain 50 ± 10% water, 6.8 × 10^{-5} milliequivalents of Na per mg. dry weight and 8.3 × 10^{-5} milliequivalents of K per mg. dry weight.

2. The data suggest that when these eggs are placed in solutions of NaCl or KCl of different concentrations (0.55–0.75%), they change volumes as osmometers with all of the water acting as a solvent.

3. Ovarian eggs of *Hyla labialis* (with follicular layers attached) contain 55 ± 10% water, 4.4×10^{-5} milliequivalents of Na per mg. dry weight and 5.4×10^{-5} milliequivalents of K per mg. dry weight.

4. The data also suggest that these eggs in solutions of NaCl and KCl change volumes as osmometers with all of the water acting as a solvent.

5. Water, Na and K can move across the membranes of both *Bufo* and *Hyla* eggs.

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STUDIES ON THE ACTION OF PHENYLTHIOUREA ON THE RESPIRATORY METABOLISM AND SPINNING BEHAVIOUR OF THE CYNTHIA SILKWORM

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There is now abundant proof, furnished particularly by Schneiderman and Williams (1954), and Shappirio and Williams (1957a, 1957b), of the presence of various cytochromes and of cytochrome oxidase in the tissues of the *Cecropia* silkworm.

In the larval stage, the cytochrome system is both intact and functional, and sensitive to cyanide and carbon monoxide. During the course of metamorphosis, however, there is a precipitous fall in the rate of oxygen consumption of the *Cecropia* silkworm, and, later, when diapause intervenes during the pupal stage, the presence of cytochromes *b* and *c* becomes indetectable, while cytochrome b^5 and cytochrome oxidase are present at low levels according to spectrophotometric and spectroscopic studies of individual tissues. These changes do not occur in the cytochrome system of the intersegmental muscles of the abdomen (Shappirio and Williams, 1957a, 1957b). The partial breakdown of the cytochrome system in the rest of the tissues, however, seems to be responsible for the striking decrease in respiratory metabolism.

A distinguishing feature of the metabolism of the diapause pupa is its resistance to cyanide and carbon monoxide. This is apparently due to the great excess of cytochrome oxidase relative to cytochrome *c* in this stage, and to cyanide and carbon monoxide being incapable of inhibiting this reserve cytochrome oxidase unless the oxygen tension is experimentally decreased to exceedingly low levels (Harvey and Williams, 1958a, 1958b; Kurland and Schneiderman, 1959). Previously, the cyanide- and carbon monoxide-insensitive character of the metabolism of the diapause pupa suggested the possibility that tyrosinase might be serving as a terminal oxidase in respiration instead of cytochrome oxidase. Sussman (1949, 1952), however, showed that this was not the case.

Since it has long ago been shown that cytochrome oxidase is immune to the action of various urea compounds (Grant and Krantz, 1942) and phenylthiourea (DuBois and Erway, 1946), it has been customary to use the latter compound, a copper-catalyst blocking agent, to inhibit the activity of blood phenolase to prevent the formation of toxic quinolic substances after surgical operation on insects. It was therefore of particular interest that the use of the compound for this purpose in some experiments on Cynthia silkworms seemed to depress their metabolism.

Preliminary studies on the oxygen consumption of *Cynthia* revealed significant decreases in the rate after treatment with phenylthiourea. Moreover, it was also noticed that treatment with this compound affected the nature of the cocoon spun later by the silkworm.

The difference in degree of respiratory metabolism, as shown by these preliminary results, suggested that the respiratory metabolism of *Cynthia,* though probably mediated by the usual cyanide- and carbon monoxide-sensitive cytochrome oxidase system, is sensitive to the action of a copper-catalyst inhibitor.

This investigation was therefore undertaken to elucidate further the depressant effect of phenylthiourea on respiratory metabolism, and the accompanying effect of this compound on the spinning behaviour of *Cynthia.*

MATERIALS AND METHODS

The present study is based on respiratory measurements of final instar larvae and pupae of the silkworm, *Philosamia cynthia.*

Phenylthiourea was introduced into the blood of the silkworm, under CO_2 anaesthesia, either by inserting it as crystals through an incision made in the abdomen, or by injecting it in a physiological solution from a hypodermic syringe.

Both the experimental and control animals were kept in glass tubes containing a strip of moistened filter paper, and were fed on privet. They were transferred to vessels of approximately 40-cc. for use with standard Warburg manometers for oxygen-consumption measurements. Each vessel was divided into two connected well-compartments by an infolding of the base. To absorb the carbon dioxide output, a loose roll of filter paper was moistened with 0.5 to 0.7 cc. of 1.5 N sodium hydroxide and placed in one of the compartments, the silkworm being placed in the other. To protect the silkworm, another strip of filter paper was placed over the moistened roll. The silkworms only very occasionally disturbed the arrangement of filter papers.

Measurements were performed at 25° C. They were usually carried out at intervals of ten minutes over a period of half an hour or longer. The rate has been expressed as $mm.^3$ oxygen per gram weight of the pupa per hour, following the procedure of Schneiderman and Williams (1953), to obviate the changeability in weight of a feeding and spinning silkworm.

RESULTS

1. *Normal changes in oxygen consumption during metamorphosis*

The maximal rate of oxygen consumption in *Cynthia* prior to metamorphosis is in the region of 1400 $mm.^3$ O_2/gm.pupal wt./hr. at 25° C. (Fig. 1).

At metamorphosis, considerable changes take place. The rate of oxygen consumption begins to fall two and a half days before the onset of spinning. It continues to fall, reaching a level of about 250 $mm.^3$ O_2/hr. at the end of spinning and of 75 $mm.^3$ O_2/hr. in the pupal stage.

The precipitous nature of this fall in the rate of oxygen consumption in *Cynthia* during the course of metamorphosis at 25° C. is in agreement with that recorded for *Cecropia* (Schneiderman and Williams, 1953). There is, however, an important difference with regard to the timing of the fall. Whereas in *Cecropia* the decrease begins just after the cocoon has been spun, the decrease in *Cynthia* begins two and a half days before spinning is initiated. Indeed, at the beginning of the spinning phase, in *Cynthia,* the rate of oxygen consumption has already fallen to half the maximal level.

Cynthia takes two days to construct a cocoon, the outer envelope being completed in 24 hours, and the loose intermediate layer and inner envelope in the next 24 hours.

2. *Inhibitory effect of phenylthiourea on oxygen consumption*

Measurements of the rate of oxygen consumption were carried out on feeding fifth instar *Cynthia* before and after treatment with phenylthiourea. The compound was inserted as crystals through an incision made in the abdomen. Though it is highly insoluble in water, being taken up at about 2 mg. per cc., it dissolves readily in *Cynthia* blood. The weights of the silkworms used ranged from 3 to 4 gm.

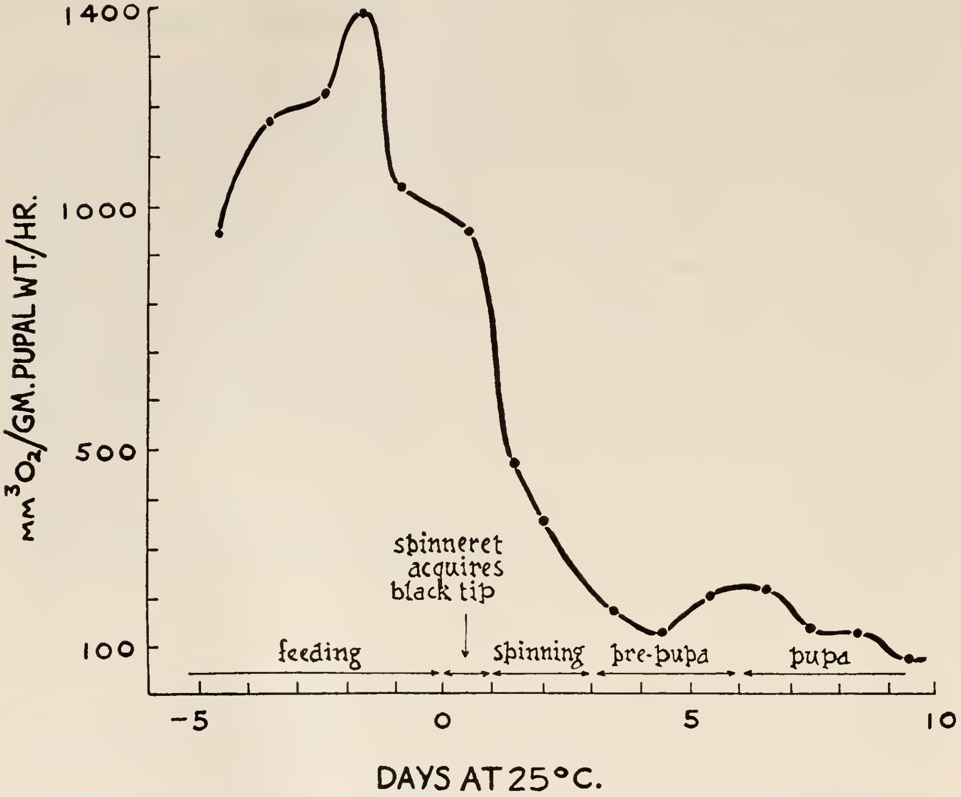

FIGURE 1. Changes in the rate of oxygen consumption at 25° C. of the *Cynthia* silkworm during metamorphosis.

Figure 2 shows the depressant action of the inhibitor at varying amounts on the rate of oxygen consumption. When 0.45 mg. was introduced in the way just described, there was a significant decrease in the rate of oxygen consumption of the treated silkworm. The rate subsequently increased without, however, reaching the pre-treatment level. At the onset of spinning the rate was about 650 mm.^{3} O_2/hr.

At 0.75 mg., the depressant action of the compound on the rate was more marked. The fall in the rate, however, was again followed by an increase, but this failed to reach a level above 500 mm.^{3} O_2/hr. before spinning was initiated. Moreover, silkworms treated with this amount of the compound were rendered incapable of constructing a normal cocoon, but they were not, however, prevented from pupating normally.

At 1.0 mg., there was a striking fall in the rate. Silkworms treated with this amount of the compound were, after a delay of several hours, liable to become completely immobilized. This induced quiescent state usually lasted about two days. This was followed by a gradual recovery which was accompanied by an increase in the rate of oxygen consumption. The rate, however, seldom reached a level above 400 mm.^{3} O_2/hr. before spinning took place. Though the usual two-day period was spent in spinning by these treated silkworms, it was observed that they failed to produce more than a limited sheet of silk.

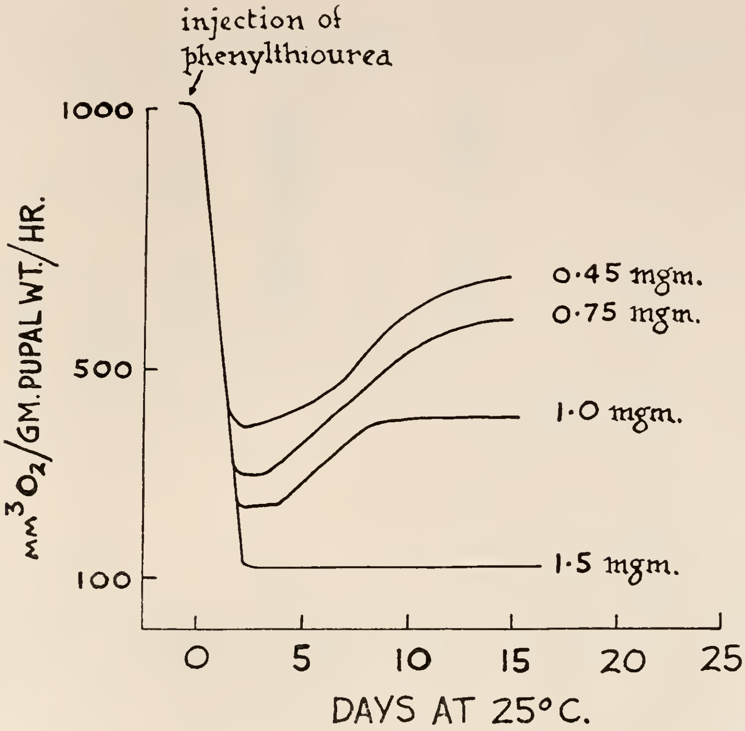

FIGURE 2. Depressant action of phenylthiourea at different concentrations on the rate of oxygen consumption of feeding fifth instar *Cynthia* larvae at 25° C.

Amounts of phenylthiourea in excess of 1.5 mg. were usually lethal to the silkworms used. They invariably brought about a precipitous fall in the respiratory rate, and so completely immobilized the treated silkworms that they seldom showed any signs of recovery.

These studies indicate that the depressant action of phenylthiourea on the rate of oxygen consumption in *Cynthia* is proportional to its concentration in the blood and tissues. When amounts of less than 0.45 mg. were introduced into the blood, they also significantly lowered the respiratory rate, and the results which indicate this are considered in a later section of this paper. Moreover, it will be apparent that the *Cynthia* silkworm seems capable of reversing the depressant action of phenylthiourea on its respiratory metabolism, provided the amount of the compound administered is less than the lethal dose.

3. *Inhibition of blood phenolase activity*

Efforts were next made to determine the inhibitory effect of phenylthiourea on the blood phenolase. Though it was unlikely that the site of the depressant action on the respiratory rate was the blood phenolase, it was nevertheless desirable to estimate the ability of this compound to inactivate a copper-catalyst which was known to be present in the blood and probably the tissues.

Phenolase (tyrosinase) is distinguished from other copper enzymes by its ability to catalyze the insertion of an hydroxyl group into monohydric phenols and the oxidation of the resulting o-diphenols to their corresponding o-quinones (Dawson and Tarpley, 1951).

It is generally accepted that the enzyme is present in the blood of insects, and that when the blood is exposed to air it changes to a brown colour owing to the enzymatic oxidation of polyphenols to form melanin-like substances. The addition of a diphenol substrate such as dihydroxyphenyl-alanine to the blood of an insect results in the rapid formation of melanin, due to the reaction being catalyzed by the phenolase. If phenylthiourea is also added, melanin is not deposited. The inference to be drawn from this is that this compound in blocking the activity of the phenolase suppresses the development of melanin.

Most inhibition studies carried out with the enzyme have been performed with substances known to form stable complexes with copper. Among the substances used, the thioureas seem to be very effective blocking agents of phenolase (DuBois and Erway, 1946). Little is known at the present time, however, as to how the copper is bound within the enzyme, or what precisely is its role in phenolase activity (Dawson and Tarpley, 1951). It is likely, however, that phenylthiourea exerts its inhibitory effect on phenolase by reacting with the copper component of this enzyme, as it does with the copper which catalyzes the oxidation of ascorbic acid (DuBois and Erway, 1946).

Dawson and Tarpley (1951) have directed attention to the fact that the oxidation of di- and polyhydric phenols can be brought about in other ways. For example, these phenols can be oxidised by hydrogen peroxide in the presence of peroxidase, and aerobically in the presence of cytochrome *c* and cytochrome oxidase. Presumably, this type of oxidation cannot be inhibited by a copper-catalyst blocking agent such as phenylthiourea which has no effect on the enzymes concerned. Copper alone, or in complex form with non-specific proteins, peptides, or amino acids, can also apparently catalyze the aerobic oxidation of these phenols. There is strong evidence, however, in support of the conclusion that in insects a blood phenolase (tyrosinase) is responsible for the formation of melanin from polyphenols (Mason, 1955).

The following tests were carried out to permit an estimation of to what extent the blood phenolase of *Cynthia* could be inhibited by the introduction of phenylthiourea. Silkworms weighing about 3 to 4 gm. were treated with amounts of either 0.45 mg. or 1 mg. of the compound by the method already described. Samples of blood were taken from these silkworms 24 hours later. To the samples was added a solution of dihydroxyphenyl-alanine so that 1 cc. of the final solution contained 1 mg. of the phenol substrate. Figure 3 illustrates samples of blood from silkworms treated or untreated previously with the inhibitor, and also samples to which the substrate has been added.

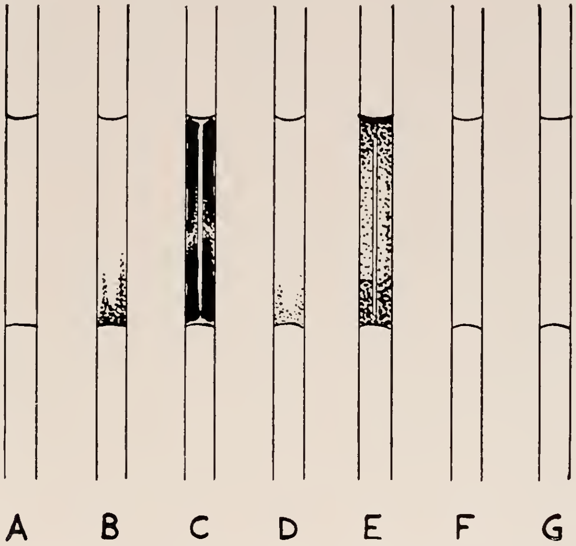

FIGURE 3. Inhibitory effect of phenylthiourea on melanin formation in *Cynthia* blood. For details see text. A, solution of diphenol substrate; B, untreated blood; C, untreated blood incubated with the diphenol; D, blood from silkworm injected 24 hours previously with 0.45 mg. of the inhibitor; E, same as D incubated with the diphenol; F, blood from silkworm injected 24 hours previously with 1 mg. of the inhibitor; G, same as F incubated with the diphenol. Drawings taken from photographs of the results of this experiment.

Though distinctions could be drawn after a few hours between the different samples according to the intensity of melanin development, they became more clearly marked at the end of 24 hours (Fig. 3). There seemed to be no further increase in the intensity of melanin deposited after about 30 hours.

It is concluded from a series of tests of the kind illustrated in Figure 3 that whereas 0.45 mg. of phenylthiourea partially blocked the activity of the blood phenolase, 1 mg. completely blocked it. Moreover, the inference to be drawn from the results of these tests is that the inhibitor is inactivating the enzyme *in vivo*.

4. *Effect of changes in the metabolic rate on cocoon spinning*

To elucidate further the effect of phenylthiourea upon the spinning behaviour of *Cynthia*, studies were next made on the depressant action of this compound on respiratory metabolism during the spinning period.

Table I

Relationships between the depressant action of phenylthiourea on the rate of oxygen consumption at the beginning of spinning and the types of structures spun. For details see text.

Serial no.	Mg. of agent injected 24 hr. previous to spinning	mm.^{3} O_2/gm. pupal wt./hr.			Types of cocoon spun
		Uptake at the beginning of spinning	Uptake at the end of spinning	Range	
P 6	nil	740	210	520	Normal
P 8	0.1	700	260	440	Two-layered and closed
P 1	0.1	700	280	420	Two-layered and closed
P 4	0.3	520	280	240	Two-layered and open
P 9	0.4	500	250	250	Hammock-shape
P 7	0.5	525	265	260	One-layered and open
L 2	0.6	490	290	200	Hammock-shape
P 2	0.7	385	210	175	Hammock-shape

An important symptom of metamorphosis in *Cynthia*, as already described, is the striking fall in the rate of oxygen consumption, which begins two and a half days before the onset of spinning. The injection of phenylthiourea into silkworms during this pre-spinning period would be expected therefore further to depress the already decreasing rate of oxygen consumption. It is also reasonable to assume that this fall in the rate of respiration reflects the progress in the series of events of metamorphosis which is triggered off by the "pupation" hormone (Williams, 1947). The timing of the onset of spinning is therefore already under hormonal control, and the insertion of phenylthiourea into the blood just prior to the spinning period is unlikely to influence it.

Accordingly, the compound was injected in solution into *Cynthia* about 24 hours before spinning was due to begin. This stage was conveniently marked by

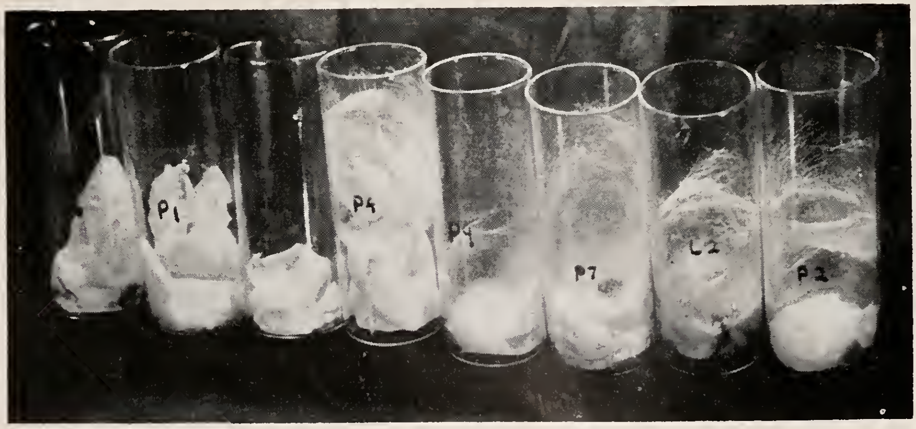

Figure 4. Series of structurally different cocoons spun by *Cynthia* after being injected 24 hours previously with varying amounts of phenylthiourea. From left to right in the photograph relative amounts of the inhibitor used were, nil, 0.1 mg., 0.1 mg., 0.3 mg., 0.4 mg., 0.5 mg., and 0.7 mg.

the tip of the spinneret turning black. The amounts of the compound introduced into the blood in these studies ranged from 0.1 to 0.7 mg.

The results in Table I illustrate a relationship between the depressant action of phenylthiourea on the rate of oxygen consumption at the beginning of spinning and the types of structures spun. It seems that the extent to which the rate of respiratory metabolism is decreased at the beginning of spinning determines the type of structure spun (Fig. 4).

DISCUSSION

1. *Depressant action of phenylthiourea on respiration*

The results of this investigation show that the influence of phenylthiourea on the respiration of *Cynthia* is one of depression, which is proportional to the concentration.

During the course of normal metamorphosis in silkworms there is a striking fall in the rate of oxygen consumption. A considerable fall in the rate, however, also occurs at each moult, and it is significant that when a larva is deprived of food there is an accompanying decrease in the respiratory rate (Wilson, unpublished work). It seems, therefore, as if cessation of feeding by silkworms prior to metamorphosis may be partly responsible for the decrease in the metabolic rate.

The introduction of phenylthiourea into the blood of *Cynthia* just before the spinning phase markedly depressed the rate of oxygen consumption below the normal level. This obviates the possibility that the inhibitory effect of phenylthiourea on respiration is simply due to this compound halting feeding. The evidence instead converges in support of this compound exerting an inhibitory action upon some site in the respiratory chain of *Cynthia*. It seems unlikely, however, from previous work that the site of the depressant action is the cytochrome oxidase or succinic dehydrogenase systems (Grant and Krantz, 1942; DuBois and Erway, 1946).

Since the experimentally induced fall in the rate of oxygen consumption in *Cynthia* is accompanied by an inhibition of the blood phenolase activity, the possibility of this enzyme being implicated in some way with respiration, as suggested by Heller (1947), cannot be ruled out. Though potentially capable of serving as a terminal respiratory oxidase, it would, however, seem unnecessary for phenolase to perform this function in a silkworm in which there is an intact and functional cytochrome-cytochrome oxidase system. If one prefers to accept the possibility of phenolase serving as a terminal respiratory oxidase despite the presence of cytochrome oxidase, there then remains the further possibility that phenolase may be coupled with the oxygen which is supposed to diffuse from the tracheoles and across the short distances to the adjacent tissues.

It was suggested long ago, however, that the depressant action of urea derivatives on respiration is exerted on a part of the respiratory chain involving DPN, and not on the oxygen terminal part of the chain (Grant and Krantz, 1942). This is still believed to be the case (Slater, 1958).

Morton (1958) has recently drawn attention to the wide distribution of quinone compounds in tissues, and Slater (1958) has suggested how these compounds may be involved in oxidative phosphorylation. The evidence up till now is in favour of this, and it is therefore tempting to suggest that if the quinones in question are derived from quinol compounds only in the presence of an active phenolase-type enzyme, then blocking this catalyst would be expected to have an inhibitory effect on oxidative phosphorylation. To speculate further than this is unlikely to be of much value because there are still many gaps in our knowledge about the processes involved in oxidative phosphorylation.

2. *Relation of metabolic rate to spinning behaviour*

It will be seen from the results shown in Table I that the differences between the cocoons spun by *Cynthia* after treatment with varying amounts of phenylthiourea are qualitative. This obviates the results being attributed to a general muscular debility. Moreover, during the spinning period the treated silkworms were as active as the controls. This recalls the observation of Van der Kloot and Williams (1954) that *Cecropia* retains its normal activity when exposed to carbon monoxide yet at the same time is rendered incapable of spinning a cocoon.

The results of the present work show that the type of cocoon spun depends on the extent that the rate of oxygen consumption is depressed at the beginning of the spinning phase by phenylthiourea. Since the cocoon is the end-result of a complicated pattern of neuromuscular activity, any deviation from normal in the structure of the cocoon may be taken as reflecting a change of behaviour. It is therefore reasonable to conclude that quantitative differences in the metabolic rate are coupled in the silkworm with differences in behaviour which are qualitative. It would be interesting to learn to what extent this principle can be extended to other forms of behaviour, whether instinctive or otherwise.

SUMMARY

1. A symptom of the metamorphosis of *Cynthia* is that the precipitous fall in the rate of oxygen consumption begins two and a half days before the spinning period.

2. The rate of oxygen consumption is reduced to half the maximal rate of 1400 mm.^{3} O_2/gm. pupal wt./hr. when spinning begins. It continues to fall throughout the spinning phase and reaches a level of 75 mm.^{3} O_2/hr. in the early pupal stage.

3. Phenylthiourea has a pronounced depressant action on the rate of oxygen consumption, the depression being proportional to the amount of this compound introduced into the blood.

4. The decrease in respiration brought about by phenylthiourea coincides with the inhibition of blood phenolase activity. Possible sites of the depressant action of phenylthiourea on respiration are discussed.

5. When the rate of oxygen consumption is depressed to various levels at the beginning of spinning a series of qualitatively different cocoons is produced.

6. It is concluded that the pattern of spinning behaviour is delicately tuned to the metabolic rate.

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ON THE EMBRYONIC DEVELOPMENT OF THE SEA URCHIN ALLOCENTROTUS FRAGILIS[1]

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Allocentrotus fragilis, thanks to its brightly colored test and whitish short spines, has been termed the most beautiful of the sea urchins. Perhaps because of the fragility of the almost paper-thin test and the depths at which this sea urchin lives there was no study of the species before that of Jackson in 1912. He gave it the name *Strongylocentrotus fragilis*. In 1943 Mortensen, in his revision, assigned to the species a new generic title, *Allocentrotus* (Boolootian *et al.*, 1959).

The first specimens taken in any quantity at the Pacific Grove station were dredged from depths of 80 to 100 fathoms, temperature approximately 8° C. Although samples were taken throughout the year, I have found ripe eggs only in February and March. Animals dredged in mid-April showed that complete spawning had taken place, with the exception of one male which yielded a little active sperm. The animals lived very well for at least two weeks in aquaria of the laboratory in which the temperature of the running sea water was approximately 14° C. Eggs and sperm were taken from these animals from time to time. The specimens used were from 50 to 80 mm. in diameter. Only one of those taken during the three seasons equaled in diameter the largest recorded by Clark (1948), namely a little over 100 mm. The test of this specimen is in the collection of Dr. J. Wyatt Durham of the Department of Paleontology, University of California, Berkeley.

Eggs and sperm were first obtained by opening the animals and putting the gonads into dishes of sea water into which the sex products quickly escaped. Later, in order to avoid waste, the electrical method (Harvey, 1953) was used and found to be very satisfactory. The eggs are very light in color, so that, as they stream out of the ovary they may at first be mistaken for sperm. The diameter of the egg is approximately 110 μ which is midway between the dimensions of the eggs of the two shore forms, *Strongylocentrotus purpuratus* (78 μ) and *Strongylocentrotus franciscanus* (140 μ). The fertilization membrane closely invests the egg, being in distance from the egg's surface $\frac{1}{16}$ the diameter of the egg (Fig. 1). In contrast the fertilization membrane of *S. purpuratus* is removed from the surface of the egg $\frac{1}{6}$ the diameter of the egg.

At a given temperature, the eggs of *Allocentrotus fragilis* divide at exactly the same rate as those of the two shore forms *Strongylocentrotus purpuratus* and *S. franciscanus*. The temperature for successful development of *Allocentrotus fragilis* must be kept at 15° C. or lower (Moore, 1959) (Figs. 1–4). There is, however, some variation, the eggs of an occasional individual segmenting normally at 16°

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[1] Identified for me by Dr. J. Wyatt Durham.

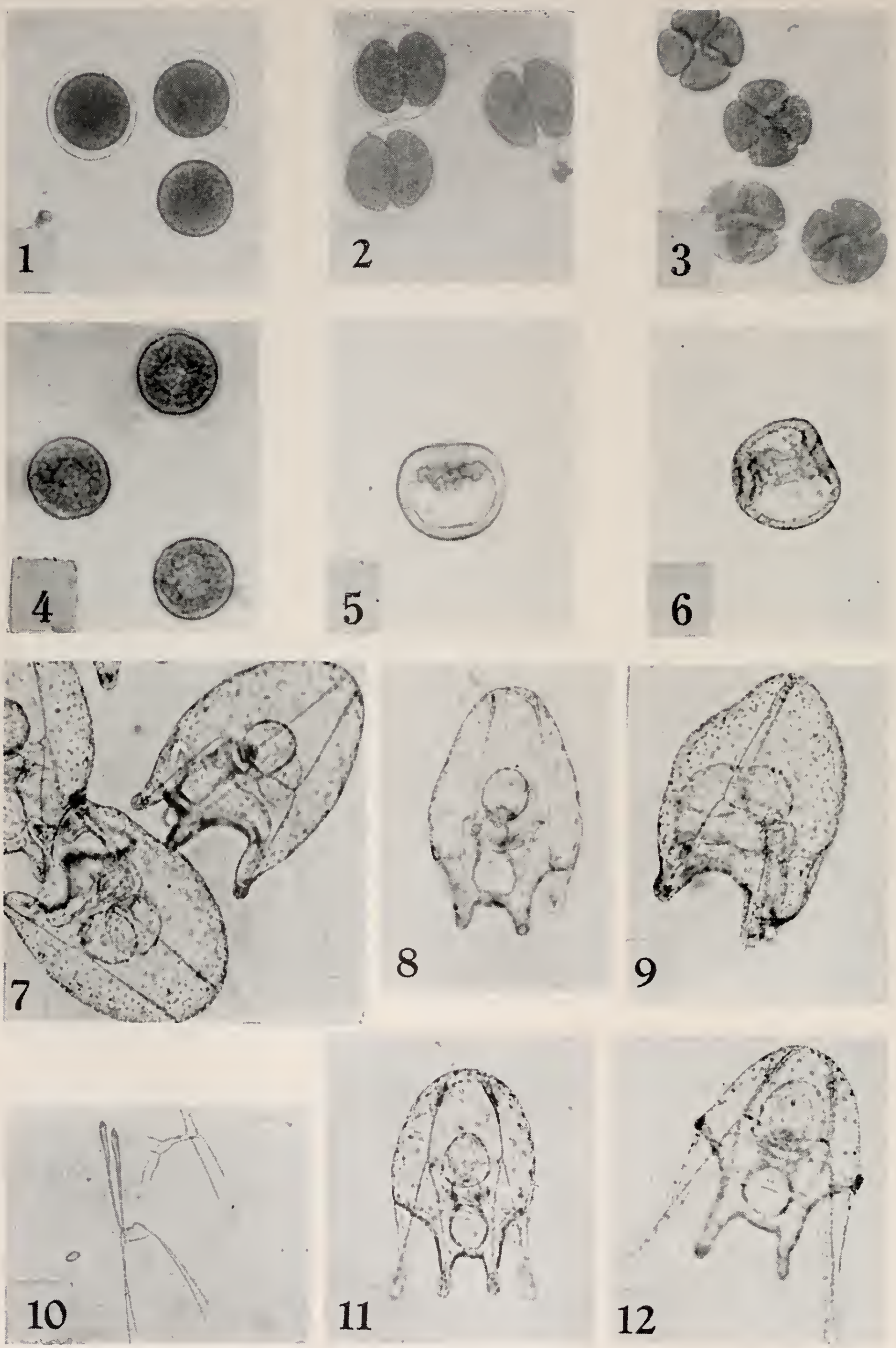
or even 17° C. while others require 14° C. or lower for successful development.

The blastulae (Fig. 5) early show flattening at the poles, in that way differing from the oval form of the same stage in *S. purpuratus*. The pluteus, which is formed three days after fertilization, resembles that of *S. purpuratus* in having short arms, but the shape of the body is somewhat cylindrical and thus differs from that of *S. purpuratus* which is conical or pyramidal in shape (Figs. 7, 8, 9). The skeleton (Fig. 10) resembles that of *S. purpuratus* in the club-shape of the body-rods, in its smoothness and in the short post-oral rods; the cross-bars are absent. In general the skeleton is thinner than that of *S. purpuratus*. An accessory post-oral rod is frequently present. In contrast to the comparatively simple structures of these two forms, the pluteus of *S. franciscanus* is more elaborate in that it is characterized by long arms, the presence of cross-bars and thorniness of the body-rods at the apex (Fig. 12).

Cross-fertilization with the two shore forms succeeded especially well in February. The eggs of *S. purpuratus* fertilized with the sperm of *A. fragilis* gave more than 50 per cent fertilization as did the reciprocal cross. The differences between the two forms in the characters of the pluteus are so slight that no study of these hybrids was made at this time. The case is different in the cross *A. fragilis* ♀ × *S. franciscanus* ♂. Here the percentage of the eggs fertilized varied between 20 and 50 per cent. Figure 11 illustrates a pluteus of this hybrid. The lengthening of the arms, the presence of cross-bars and thickening of the apical skeleton are the chief features derived from the male parent (Fig. 12). The hybrid therefore has longer arms and more complex skeleton than the maternal species by virtue of characters inherited from the male parent, and therefore follows the pattern of the cross *S. purpuratus* ♀ × *S. franciscanus* ♂ (Moore, 1943).

DISCUSSION

The deep water sea urchin, *Allocentrotus fragilis* has been taken at depths of 40 to 417 fathoms from Vancouver Island to Pt. Santa Eugina in Lower California (Clark, 1948). The species thus can thrive at a very low temperature and in the absence of light. The short breeding season, February and March, raises the question of the triggering of spawning, which takes place promptly at the end of March or beginning of April. This is the record of three successive seasons at the Pacific Grove station, and leads one to believe that spawning is, for some reason, associated with the onset of spring. Increase of light or temperature as causative factors would seem to be excluded because of the conditions at the great depths at which this species flourishes. Pertinent to the question of the triggering of spawning is the fact that ripe individuals have been kept in laboratory aquaria at a temperature of approximately 14° C. for two weeks or longer without their showing any evidence of spawning. During the time the animals retained their spines, they yielded eggs and sperm. Thus, a sudden increase in temperature and abundant light did not show any effect of triggering the spawning of ripe individuals. As to the cause of spawning in the natural habitat, there is the possibility in the spring flowering of the plankton algae. Thorson (1946) has suggested that these algae may, by their fall to the bottom, yield algal extracts which furnish the chemical trigger for spawning. There remains the possibility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FIGURES 1–12.

that this animal, as a character of its being, has an annual periodicity which is independent of environmental factors.

The close relationship of *Allocentrotus fragilis* to the two shore forms of *Strongylocentrotus* is indicated, first, by the exact time relations of development, which are precisely the same in the three species, and by the fact that crosses can easily be made between *Allocentrotus fragilis* and the two species of *Strongylocentrotus.* The inheritance of paternal characters in the cross *Allocentrotus fragilis* ♀ × *S. franciscanus* ♂ proves that we have here true fertilization and not parthenogenesis.

It gives me pleasure to record my cordial thanks to Mr. Tom Fast who made the initial discovery of the bed of *Allocentrotus fragilis* at Pacific Grove in February, 1957, and who gave me the entire number taken at that time. Subsequent hauls were made by junior members of the Station staff who very graciously furnished me with specimens needed for my work.

Summary

1. Ripe eggs and sperm of *Allocentrotus fragilis* were obtained during February and March, from animals taken at depths of 80 to 100 fathoms.

2. Fertilization was approximately 100 per cent, and development was normal at a temperature of 15° C. or lower.

3. The rate of development in *Allocentrotus fragilis* is identical to that of the two species of *Strongylocentrotus* at this locality.

4. The pluteus has a characteristic form which distinguishes it from those of *S. purpuratus* and *S. franciscanus.*

5. Cross-fertilization between *Allocentrotus fragilis* and the two forms of *Strongylocentrotus* was accomplished, and in the progeny of *A. fragilis* ♀ × *S. franciscanus* ♂ the development of paternal characters was clearly shown.

6. As to the cause of spring spawning, increase in light and possible increase in temperature are not factors, but Thorson's idea of the occurrence of algal extracts of the plankton in early spring is suggested as a possible factor. There is also the possibility of an innate periodicity characteristic of the species.

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Figures 1 to 10 inclusive refer to *Allocentrotus fragilis.* Magnification 100 ×.

Figure 1. Fertilized egg.
Figures 2, 3. Segmentation stages, 2-cell and 4-cell.
Figure 4. Blastula before hatching.
Figure 5. Swimming blastula, 24 hours.
Figure 6. Gastrula, 48 hours.
Figures 7, 8, 9. Plutei, 5 days.
Figure 10. Skeleton of pluteus of *Allocentrotus fragilis.*
Figure 11. Pluteus of hybrid *Allocentrotus fragilis* ♀ × *Strongylocentrotus franciscanus* ♂, 5 days.
Figure 12. Pluteus of *Strongylocentrotus franciscanus,* 5 days.

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ACOUSTIC ORIENTATION IN THE CAVE SWIFTLET

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Acoustic orientation has been demonstrated in eleven families of bats of the suborder Microchiroptera (Griffin and Galambos, 1941; Galambos and Griffin, 1942; Griffin, 1958; Moehres, 1953; Moehres and Kulzer, 1955a, 1955b, 1957; Griffin and Novick, 1955; Novick, 1958a), in the Megachiropteran bat genus, *Rousettus* (Moehres and Kulzer, 1956; Novick, 1958b), and in the oilbird, *Steatornis caripensis* (Griffin, 1953). In each of these cases, the acoustically orienting individual produces and emits specialized clicks or pulses of sound which are reflected by intercepting surfaces such as obstacles or insect prey. The echoes from such surfaces are received by ear, interpreted, and the information used for navigation in pursuit of prey or avoiding obstacles. Griffin has called such an orientation system, based on the sounds which the navigating animal itself produces, echolocation. In the case of the fruit bat, *Rousettus,* and the oilbird, *Steatornis,* such acoustic orientation is facultative and is used to supplement vision in poor light. Among the Microchiroptera acoustic orientation is obligatory. In no known case, can these bats orient successfully by vision. While *Rousettus* and *Steatornis* probably need echolocate only obstacles and landmarks, some of the Microchiroptera have reached a high degree of specialization in tracking flying insect prey by echolocation. The occurrence of such a phenomenon among three such diverse groups suggested that acoustic orientation might prove to be common among vertebrates that fly in the dark. *Steatornis* and *Rousettus* are both nocturnal and cave-dwelling. Griffin (1953) found one chamber of a cave occupied by *Steatornis* to be totally dark at the time the birds were flying about successfully. Novick (1958b) found *Rousettus* living only in dimly lighted caves, but their ability to fly in total darkness has been shown experimentally (Griffin *et al.,* 1958). The Microchiroptera are all nocturnal and are frequently cave-dwelling as well.

Swiftlets of the genus *Collocalia* frequently inhabit caves, especially for nesting (Baker, 1934). Though their behavior in this respect is by no means uniform, many *Collocalia*-inhabited caves are known to be totally or almost totally dark. Though these birds feed diurnally and roost during the night, it seemed likely that, lacking the ability to echolocate, they would find cave flight difficult, especially in caring for their young. They would, moreover, be light-adapted each time they abruptly entered their cave, further reducing the usefulness of vision. Thus there appeared to be a strong likelihood that these birds would prove to be orienting acoustically under conditions of poor light.

In the course of an expedition which I undertook to study the orientation of the bats of the Old World tropics (Novick, 1958a, 1958b), I was able to observe the behavior of the cave swiftlet, *Collocalia brevirostris unicolor* in the vicinity of Namunukula, Uva Province, Ceylon in April, 1956. In addition, I observed swiftlets, *Collocalia* sp., in Cavite and Bataan Provinces in the Philippines between

November, 1955 and February, 1956. Griffin (1958) has reviewed the preliminary reports from this expedition.

This work was supported in part by the Office of Naval Research, the United States Public Health Service, the Sigma Xi-RESA Fund, the Belgian American Educational Foundation, and Harvard University. During this period, I was a Fellow of the National Institute of Neurological Diseases and Blindness and a Research Fellow in Biology at Harvard University. I gratefully acknowledge the aid given me by the personnel of the United States Navy and Air Force in the Philippines and by the personnel of the American Embassies in the Philippines and Ceylon. I am most deeply indebted to Major W. W. A. Phillips of Namunukula, Ceylon, for his help in observing, capturing, identifying, and studying the swiftlets, and for the kind hospitality which he and Mrs. Phillips extended to me. His many years of devoted study of the birds (and mammals) of Ceylon proved invaluable. Dr. D. R. Griffin of Harvard University not only drew my attention to the question of orientation in *Collocalia* but gave me advice and support of every description.

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METHODS

The swiftlets were captured on several occasions in the Philippines and Ceylon in fine-mesh mist nets draped over cave entrances. Birds were caught both entering and leaving the caves. In Ceylon, *Collocalia* were recorded in their natural environment and in the laboratory, using an Amplifier Corporation of America Magnemite, model 610-E, portable tape recorder and crystal microphone. While the results fall short of ideal, more satisfactory equipment was not available. Two birds were also recorded in captivity, using a battery-operated 640 AA Western Electric condenser microphone with cathode follower, a custom built amplifier, two Krohn-Hite variable band-pass filters, models 310-A and 310-AB (total slope 48 db/octave), and a Dumont oscillograph, model 304H. The oscillograph screen was photographed with a Bell and Howell motion picture camera, model 70-DA. The birds were recorded in flight in partial light and in the dark. One was also recorded escaping from a net placed near the microphone.

In determining the birds' dependence on vision and hearing, cotton pellets fixed in place with collodion were used for blindfolding and ear plugging. These proved satisfactory and relatively easy to apply and remove without significantly injuring the bird.

The birds in Ceylon were identified in life by W. W. A. Phillips. In deference to the prevailing Buddhist religion, none were killed. The literature on the natural history and taxonomy of *Collocalia* is scattered but some interesting comments can be found in Baker (1934), Manuel (1937), Mayr (1937), Lack (1956), and Medway (1959).

OBSERVATIONS

The bulk of the observations herein reported concern a colony of *Collocalia brevirostris unicolor* nesting in Hindigalle Cave, Hindigalle Group, Namunukula, Ceylon. This cave is just below the summit of a hill and has a main entrance that is about four feet high and perhaps ten feet across, as well as several smaller openings along the same side of a roughly rectangular chamber. The walls and ceiling are irregular rock with few niches or crevices. There is a dirt floor. The cave, on this occasion, was damp but had no running water. It consists of a single chamber about forty feet deep, twenty-five feet wide and from five to ten feet high, and contained, at that time, several dozen nests on the ceiling at its inner, most dimly lighted end. Some of these nests looked old and neglected and may have been the remnant of the previous season. On April 5, 1956, the majority were occupied by one or two eggs or by one or usually two young, ranging in age from newly hatched to almost adult size. The nests were of uniform architecture having a large foot attached to the ceiling with an underslung bowl-shaped shelf. The basic salivary structure was reinforced with bits of moss-like material.

The light in the cave was sufficient for me to make out gross detail even while I was light-adapted. From a position near the nests, the light measured 100 units on a Weston light meter facing the entrance and 0.1 unit facing in toward the darkest corner. Standing immediately inside the principal entrance and facing inward, the light registered at 0.8 unit. This, of course, would vary with the time of day and the weather. The Weston light meter measures in candles/sq. ft. Unfortunately this is a reflected light meter.

The swifts proved reluctant to enter while I was inspecting the cave but entered readily after I had secreted myself in a corner. The birds could be seen flying about outside in pursuit of insects, and could be heard making an occasional high pitched call. As one or more flew through the cave entrance, however, they abruptly began to emit relatively low pitched clicks at a rate of a few per second. These clicks seemed uniform in quality but varied in repetition rate as the birds flew about. The rate increased as the birds reached the darker end of the cave and reached its highest point as they circled the nests and landed. In each case, when a bird turned toward the entrance to leave the cave it ceased clicking though it still had the full length of the cave to traverse.

I was able to clip a small microphone to one of the occupied nests in the center of the group of nests and, using a long cord, withdraw to a corner with the tape recorder. Though the birds were clearly disturbed by my presence and by the noise of the running recorder, they entered the cave and flew about often enough for me to record their vocalizations.

At a second location near Namunukula, Ceylon, I was able to observe nesting swiftlets enter a large, high cleft in a cliff face to tend their nests. I could not approach the nests in this case and recorded the birds as they flew through the cleft at a distance of 50 or 60 feet from me and the microphone. This cleft was nowhere dark but was quite dimly lighted to my light-adapted eyes.

Three swiftlets were captured at Hindigalle Cave and carried to a temporary laboratory which was a room about 25 feet long, 12 feet wide and about 8 feet high at the walls, with a peaked and beamed ceiling, rising some four or five feet to its apex. The walls were light colored; one wall consisted of windows. The beamed ceiling was of very dark wood. When the birds were released in this room in good light they flew silently unless they rose above the level of the painted wall into the darker ceiling peak. There they produced clicks resembling those heard in the cave. When the blinds were drawn over the windows, the birds clicked more often. In the dark or very dim light, at night, they clicked continually as long as they flew about but not when roosting. They clearly preferred not to fly in poor light and usually roosted quickly, taking flight again only when forced to do so. When the eyes of one bird were thoroughly blindfolded, the bird flew slowly about the room with an appearance of caution, following closely the contours of the walls and ceiling and clicking continually. The ears of one bird were plugged and, in the dark, this bird flew in a bewildered manner and roosted whenever possible. It appeared not to crash into obstacles as bats under similar conditions do but to flutter in a cautious manner until it came up against an obstacle and then catch hold. This bird was very reluctant to fly when deaf.

The sounds produced by these birds resemble those made by a child's rapidly rotated ratchet toy. There is a principal frequency of about 4–5 kc. accompanied by many overtones. Because of the differential sensitivity of the microphones used, these records cannot be analyzed too rigorously for the complete frequency spectrum. The recorded sounds were initiated by a high amplitude portion of relatively pure frequency of 2–6 msec. duration, followed by a low amplitude portion of highly complex wave form lasting for up to 30 msec. or more. The high amplitude portion was almost surely a direct record of the bird-emitted sound but its apparent duration and amplitude depended upon the bird's orientation relative to the microphone and its distance. The low amplitude portion may well have included echoes from adjacent surfaces. The repetition rate observed varied considerably too. In no case was a sequence of more than 2–3 seconds recorded at a favorable signal:noise ratio. In one 2-second sequence in the cave, 15 pulses occurred—a rate of 7.5/sec. In another long sequence, the rate was 6/sec. In others the rate varied from almost 10 to about 5.5/sec. Within these series, the rhythm was not regular but the pulses were apparently spaced from 60 to 300 msec. apart. The long intervals may well have represented a missing click (when the bird turned away from the microphone). These records were made of birds circling their nests when, by ear, their repetition rate was most rapid. Further studies of these birds are required to measure frequency, duration, repetition rate, and rhythm more accurately.

In the Philippines, I had occasion to observe a number of colonies of *Collocalia* sp. Only one specimen was collected and preserved. R. A. Paynter of the Museum of Comparative Zoology of Harvard University was reluctant to make a positive identification of a single specimen of this difficult group. In any case, these colonies were observed at Cabag Cave, Luksuhin, Silang, Cavite Province; a coastal cave south of Ternate, Cavite Province; and a great number of small tidal caves and overhangs along the coast west of Mariveles, Bataan Province, Luzon. These swiftlets are very abundant in central Luzon. In every case, the cave which they occupied was dimly lighted or well shaded, never fully lighted nor totally dark. In some cases, the nests were on the ceiling; in others the nests were on the walls at about 6 to 8 feet above the floor, but in all cases, the nests were so placed that they were shaded from the entrance of the cave and in the darkest locations. I was able to observe closely a colony in an abandoned, man-made tunnel near Mariveles, Bataan. The tunnel was well lighted throughout, during the day, by sunlight but at the rear, behind large steel ceiling beams, I found several clusters of nests, some of which contained one or two eggs or young of various stages of development. The nesting corners were dim but not dark. I could examine the nests without artificial light. These birds never produced any audible vocal clicks in the tunnel or in any of the other caves I visited. Nor could I detect any inaudible utterances with a custom built pulse detector and Rochelle salt crystal microphone (Griffin and Novick, 1955) sensitive to frequencies between 10 and 100 kc. On one occasion, a single bird, somewhat differently marked from the rest, clicked like *C. brevirostris unicolor*. I was unable to capture or relocate this individual. At night the terrain outside this tunnel was floodlighted to prevent the theft of quarry equipment. The tunnel was dark but not totally so. If the birds were disturbed (and a bright light was the most effective way of doing so) they flew out and circled the outside lights for long periods before returning to their roosts one by one. No clicks were observed. Next to the occupied tunnel on one side was a similar but better lighted tunnel which had only a few swiftlet nests in dim corners. On the other side was an almost totally dark tunnel that could be entered easily but had no swiftlets in residence or any evidence of swiftlet occupation in the past.

DISCUSSION

The occurrence of acoustic orientation and echolocation in *Collocalia brevirostris unicolor* is thus established to a reasonable certainty. These birds emit clicks of a design resembling those of *Steatornis* and *Rousettus*. These clicks are emitted only in poor light and the repetition rate increases as the light decreases. This relationship was observed in the cave, where the repetition rate increased as the bird flew deeper into the cave, and in the laboratory, where the rate increased not only with decreased direct light but as the background became dark (the dark-painted peaked ceiling). Thus there appears to be a reciprocal relationship between dependence on vision and on hearing for orientation. Flight in the dark or blindfolded is cautious but competent. Flight when the ears are plugged is incompetent in the dark.

It would appear, further, that echolocation is reserved by *Collocalia* for flight in caves. These birds are all diurnal and give no evidence of producing an echolocating type of sound while in flight in the daylight. In nature, it is only when they enter their caves that the typical sounds are emitted. Obviously, cave dwelling imposes special needs on a flying vertebrate. These birds must emerge to find sufficient food. Their diurnal hunting and flight are well facilitated by vision. When they re-enter their caves, however, they are not only light-adapted but, of course, are in dim light or even total darkness (Baker, 1934; Rabor, personal communication, quoted in Griffin, 1958). Thus, to find their nests without accident, they must supplement vision with an orientation system independent of light. Just such a system has been evolved as well by three other groups, *Steatornis, Rousettus,* and the Microchiroptera, all of which fly in darkness. A satisfactory orientation system for flight in darkness must be independent not only of light but of touch for, of course, the animal is out of contact with its physical surroundings except for the air. The information-carrying energy must move rapidly relative to the animal's movement, for otherwise timely and precise information would be lacking. Smell is unlikely to suffice though it may supplement echolocation to the extent of identifying the goal (nest, roost, edible fruit, etc.) when the animal has reached its immediate vicinity; otherwise odors not only move too erratically (depending upon air currents) but are not, of course, given off by all dangerous obstacles. Dependence on random sounds alone for informative echoes would be haphazard and insufficiently precise, just as urban men cannot depend on random light at night for finding their way on streets or in automobiles. So the system which appears to fit the logical needs is, indeed, the system which has evolved repeatedly—the dependence upon the echoes of self-produced sounds to inform the flying animals in the dark about their surroundings.

Not all species of *Collocalia* enter deep caves. Many nest routinely in shallow caves or under overhangs. I should speculate that not all species of the genus are capable of acoustic orientation and that this lack restricts their nesting sites to those that are adequately lighted. Further observations on *Collocalia* nesting and orientation are clearly necessary.

In addition, further studies of the accuracy of *Collocalia* echolocation, the mechanism by which these sounds are produced, and the versatility of the system are indicated.

CONCLUSIONS

1. The cave swiftlet, *Collocalia brevirostris unicolor,* orients acoustically in poor light or when blindfolded but orients visually in good light.

2. In nature, these swiftlets orient acoustically when they enter and fly about their dimly lighted or dark caves.

3. They emit sounds of characteristic pattern of about 4–5 kc., with an initial high amplitude portion of about 2–6 msec. duration followed by a long, low amplitude portion of undetermined significance. The maximum repetition rate appears to be about 5–10 clicks/sec. The repetition rate varies inversely with the amount of light and increases when the bird encounters obstacles.

4. The echolocation system of the cave swiftlet resembles very closely that of the oilbird, *Steatornis,* and the fruit bat, *Rousettus.*

5. Echolocation appears to be common among vertebrates that fly in darkness. Echolocation in *Collocalia, Steatornis,* and *Rousettus,* however, differs from that in the Microchiroptera not only in the design of the outgoing pulses but by coexisting with accurate, functional vision.

6. In view of the failure to elicit evidence of echolocation in a Philippine species of *Collocalia* and the habit of many species of *Collocalia* of nesting in shaded but well lighted sites, echolocation may have evolved within the genus *Collocalia.*

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ON THE PRESENCE OF MYOGLOBIN AND CYTOCHROME OXIDASE IN THE CARTILAGINOUS ODONTOPHORE OF THE MARINE SNAIL, BUSYCON[1]

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Early studies of cartilage metabolism revealed the tissue to be almost completely devoid of aerobic oxidative metabolism (Kuwabara, 1932; Bywaters, 1937). That aerobic processes do occur, however, was subsequently shown by Boyd and Neuman (1954), who found chondroitin sulfate synthesis to be accompanied by significant oxygen utilization in chick embryo cartilage, and by Person and Fine (1959a, 1959b), who more recently demonstrated cytochrome oxidase and succinoxidase activity in invertebrate and vertebrate cartilages from very young animals. Additional evidence for the existence of aerobic processes in an invertebrate cartilaginous tissue will be presented in this report.

It was observed initially (Lash, 1959) that the odontophore, a cartilaginous structure which supports the radula of the whelk, *Busycon canaliculatum,* was colored red. On analysis, the red color was found to result from the presence of myoglobin in the tissue. The myoglobin was very similar to that found by Ball and Meyerhof (1940) in the radular musculature of the same animal. Additional studies revealed that the tissue possessed readily demonstrable cytochrome oxidase activity. In this paper we present a spectrophotometric characterization of the cartilage myoglobin and its pyridine hemochrome, and evidence for the existence of cytochrome oxidase activity in homogenates of the tissue.

MATERIALS AND METHODS

Odontophores were obtained by knocking off the hard calcareous shell of the animal and exposing the proboscis. The proboscises were cut off, the odontophores dissected out, carefully trimmed of adherent muscle, and placed on aluminum foil in a beaker of cracked ice. Complete removal of muscle tissue from the odontophore was checked by means of a magnifying lens and also by examination of histological sections. For spectrophotometric and manometric studies the tissue was homogenized in a ground-glass homogenizer in 0.1 M $Na_2HPO_4-KH_2PO_4$ buffer, pH 7.4, or water, and used immediately. Some preparations were lyophilized immediately following homogenization in glass-distilled water.

Spectrophotometric studies were made with a Beckman Model DU Spectro-

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photometer and a Process and Instruments Recording Spectrophotometer, Model RS 3. For determinations of cytochrome oxidase activity, the oxidation of reduced cytochrome *c* by the tissue homogenate was followed at 550 mμ (Wainio *et al.*, 1951). Manometric determinations of oxygen uptake in a system limiting for cytochrome oxidase were made using hydroquinone as substrate in the presence of added cytochrome *c* (Sigma) (Eichel *et al.*, 1950).

Experimental and Results

1. *Absorption spectra of myoglobin*

The odontophore myoglobin was very soluble and easily dissolved out of the tissue by either glass-re-distilled water, or 0.1 *M* PO_4 buffer at pH 7.4. The curves presented in Figure 1 were obtained by homogenizing 236 mg. wet weight of freshly dissected tissue in 5.0 ml. of buffer at 4° C. The homogenate was spun in the centrifuge at 4° C. at 1700 × g for twenty minutes. The supernatant solution was decanted and used for spectrophotometric study. Reduction of the pigment was accomplished by the addition of sodium dithionite.

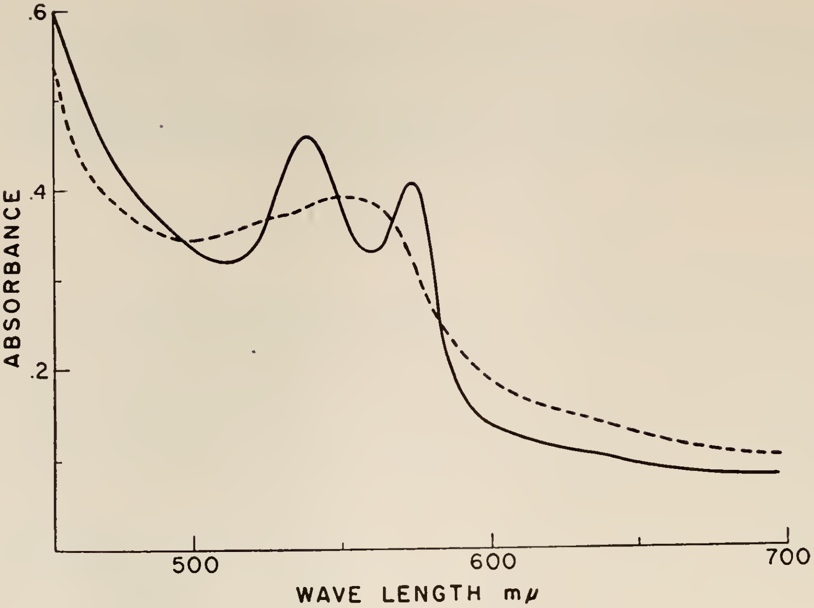
In Figure 1 are shown tracings made from original records of oxidized and reduced visible spectra, obtained in the recording spectrophotometer. The oxidized pigment (solid curve) exhibits an α peak at 574–575 mμ and a β peak at 538–539 mμ. Following dithionite reduction (dashed curve), a single broader and flattened absorption occurs at 540–565 mμ. These absorption maxima are similar to those determined (with a hand-spectroscope) by Ball and Meyerhof (1940) for the muscle myoglobin of Busycon, *i.e.*, oxidized compound, α peak, 570–580 mμ; β peak, 540–545 mμ. The discrepancies in location of the peaks may be the result of difference in instrumentation.

For study of the ultraviolet absorption of the myoglobin, the solution described above was diluted 1 : 5 with the same phosphate buffer. In Figure 2 the ultraviolet spectrum is shown. In the oxidized form (solid curve) a γ or Soret peak is present at 415–416 mμ. A broad, flat elevation encountered at 320–360 mμ is associated with the porphyrin moiety of the pigment (Barron and Flood, 1952). The sharper peak at 290 mμ is due to the presence of protein. A well-defined peak is seen at 238–239 mμ, possibly due to the presence of fatty acids in the preparation.

The spectrum of dithionite-reduced material exhibits a γ peak at 430 mμ; the remainder of the ultraviolet spectrum could not be obtained in the dithionite-reduced material because of the absorption of ultraviolet light by dithionite itself.

2. *Absorption spectra of pyridine hemochrome*

For preparation of the pyridine hemochrome of the myoglobin prosthetic group, 230 mg. wet weight of freshly trimmed odontophore were homogenized at 4° C. in 5 ml. of reagent pyridine (Merck and Co.). The homogenate was spun at 4° C. at 1700 × g for twenty minutes, and the supernatant solution decanted into a cuvette. Reagent pyridine was used in the reference cuvette. The visible absorption spectrum of the reduced hemochrome is shown in Figure 3. The α peak is located at 554–555 mμ, the β peak at 524–525 mμ. A broader and lower peak is present at 480 mμ. For determination of the ultraviolet absorption, shown in Figure 4, the pyridine supernatant described above was diluted 1 : 12 with addi-



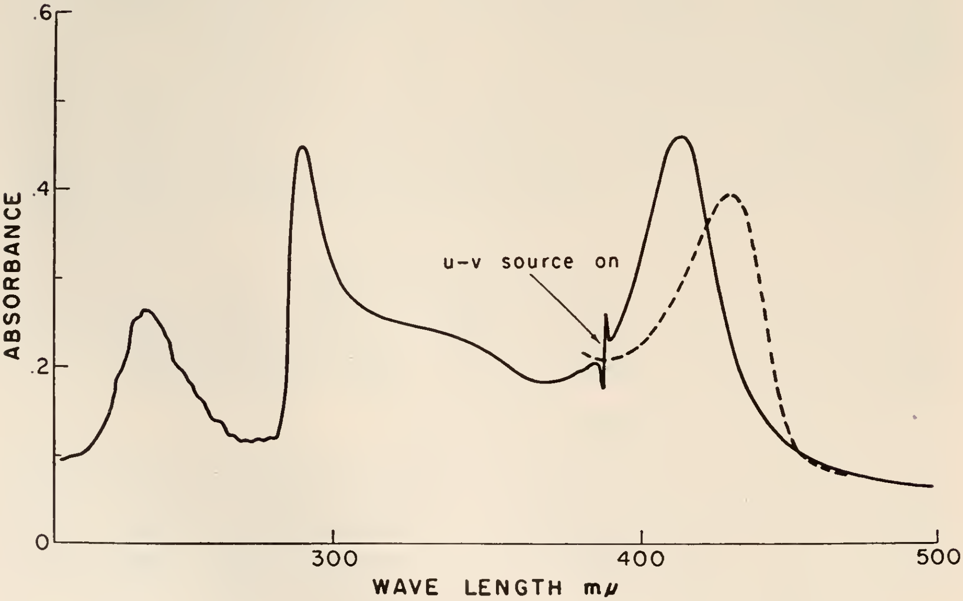
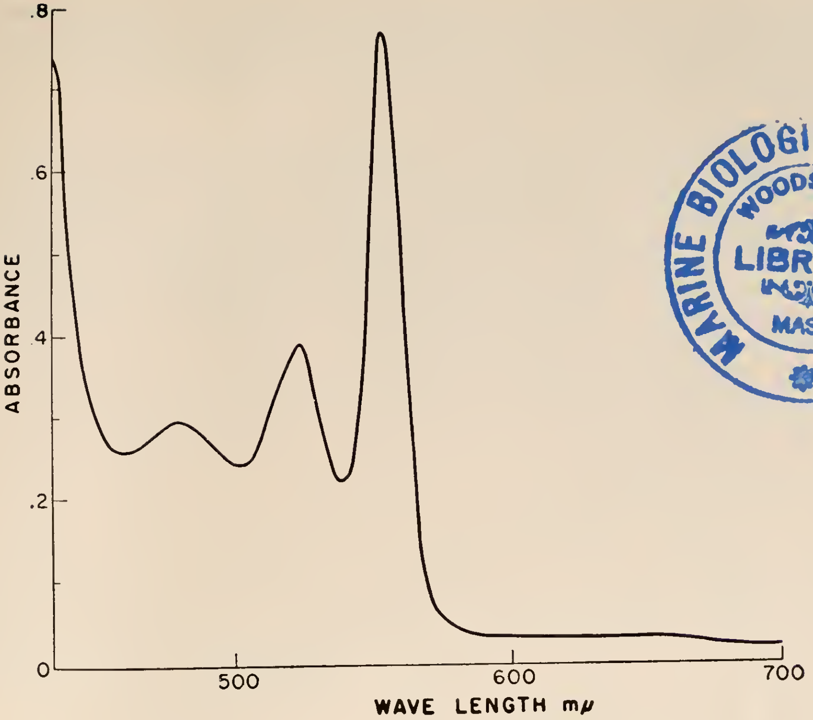


Figure 1 (above). Visible absorption of odontophore myoglobin. Solid curve: oxidized pigment; dash curve: dithionite-reduced pigment.

Figure 2 (below). Soret and ultraviolet absorptions of odontophore myoglobin. Solid curve: oxidized pigment; dash curve: dithionite-reduced pigment.



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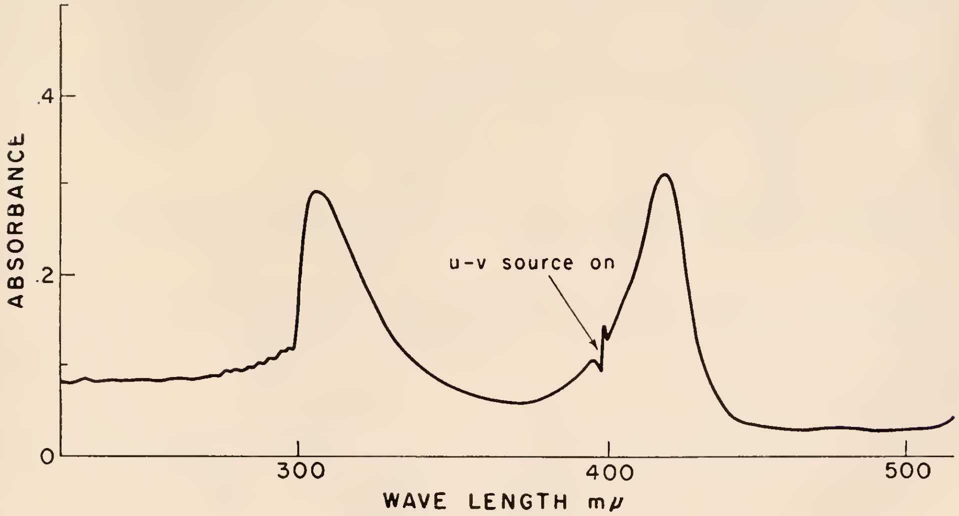

FIGURE 3 (above). Visible absorption spectrum of pyridine hemochrome prepared from odontophore myoglobin.

FIGURE 4 (below). Soret and ultraviolet absorptions of pyridine hemochrome prepared from odontophore myoglobin.

tional reagent pyridine. The γ peak is located at 417–418 mμ. The peak located at 290 mμ in the aqueous extracts is now present at 306–307 mμ. The peak at 238–239 mμ in the aqueous extracts is not present in the pyridine extracts.

3. *Cytochrome oxidase activity of cartilage homogenates*

In the spectrophotometric assay, tissue was homogenized in 0.1 *M* KH_2PO_4–Na_2HPO_4 buffer, pH 7.38. The homogenate was spun at 25,000 × g for one-half hour and the sediment re-suspended in buffer. The suspension was again spun at 25,000 × g for one-half hour and the sediment again suspended in buffer. Temperature was kept at 2°–4° C. throughout these operations. In this way endogenous substrates were removed and also most of the myoglobin, so that its absorption was not sufficient to interfere with the assay. Figure 5, curve 1, shows the decrease in absorbance of reduced cytochrome *c*, produced as a result of its oxidation by the tissue homogenate. Curve 2 depicts the inhibition of the above

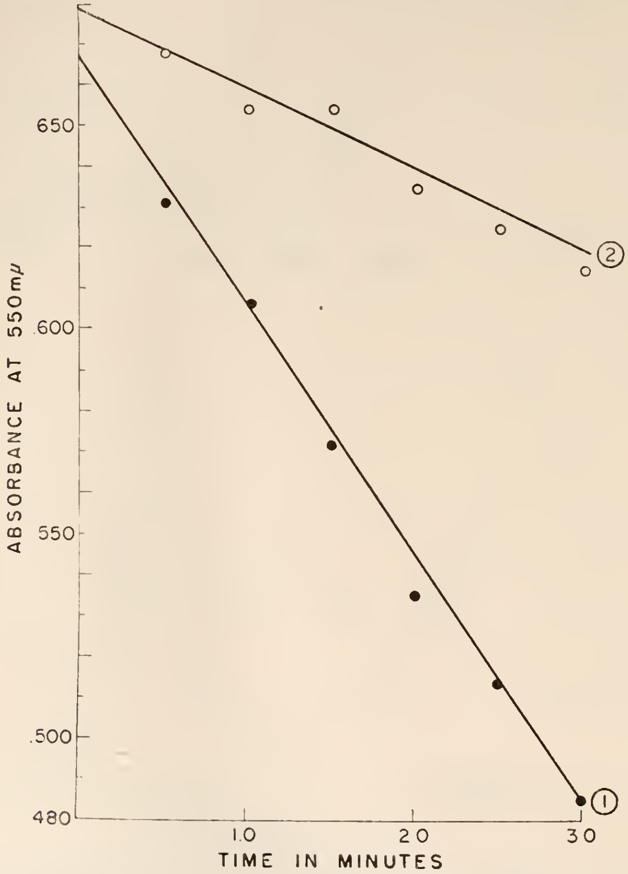

FIGURE 5. Cytochrome oxidase activity of odontophore homogenates as determined (spectrophotometrically) by oxidation of reduced cytochrome *c*. Curve (1): untreated homogenate; Curve (2): homogenate in presence of 10^{-4} *M* azide.

system by the incorporation of 10^{-4} *M* azide (final concentration). Whereas in three minutes a decrease of 0.171 absorbance units occurred in the absence of inhibitor, in the presence of 10^{-4} *M* azide, a decrease of only 0.058 absorbance units took place, or an inhibition of 66%. Similarly, 10^{-4} *M* cyanide (final concentration) produced a 75% inhibition of the cartilage oxidase activity. The cytochrome oxidase activity of homogenates prepared in the above manner was extremely labile. When kept at 0°–1° C. for 45 minutes, 50% loss in activity was noted. Homogenates in a thin-walled test tube, which were immersed in a boiling water bath for 25 minutes, were completely inactive. In a standard manometric assay using hydroquinone as substrate and added cytochrome *c*, the Q_{O_2}/(mg. dry wt.) of homogenates was 9.4 at 37° C. in an air atmosphere.

DISCUSSION

The combined presence of myoglobin and cytochrome oxidase in a cartilaginous tissue is indeed interesting. The occurrence of hemocyanin as the blood oxygen-transport pigment in the same organism makes an unusual combination of oxygen transport and respiratory pigments, as was noted earlier by Ball and Meyerhof (1940). Since the physico-chemical properties of this myoglobin have not yet been studied, its exact physiologic role is not known. However, function as an oxygen-carrier and storage pigment for the cytochrome oxidase in the tissue appears likely. We have observed that the odontophores of younger animals contain less myoglobin than do those of older animals. As the snails increase in age and size, the odontophores take on a deeper pink to red color.

The presence of cytochrome oxidase in the tissue is indicated by its ability to oxidize reduced cytochrome *c*. We have been able to detect such activity in other invertebrate and vertebrate cartilage tissues as well (Person and Fine, 1959a, 1959b). Attempts to identify the characteristic absorption spectra of the oxidase have been unsuccessful thus far.

Other studies of the polysaccharide components of the odontophore (Lash, 1959) have shown that chondroitin sulfate could not be demonstrated in the tissue.

SUMMARY

1. Myoglobin and cytochrome oxidase activities were shown to exist together in a cartilaginous tissue for the first time, in the odontophore of *Busycon canaliculatum*.

2. The absorption spectra of the cartilage myoglobin were characteristic for this class of pigments. Similarly, the absorption spectra of the pyridine hemochrome prepared from the pigment were characteristic of ferroprotoporphyrin-pyridine hemochrome.

3. The Q_{O_2} (dry weight basis) of cartilage homogenates, employing hydroquinone as substrate and added cytochrome *c*, was 9.4 in an air atmosphere at 37° C. Such homogenates were also capable of oxidizing reduced cytochrome *c*.

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ELECTRON MICROSCOPE STUDY OF THE DISTAL PORTION OF A PLANARIAN RETINULAR CELL[1]

NEWTOL PRESS

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With the increasing development of techniques of electron microscopy, ever more attention has been devoted to the study of the fine structure of photoreceptors, the details of which are beyond the limit of resolution of the light microscope. One such structure is the visual receptor of the eye of the planarian, *Dugesia tigrina.*

The classical study which has served as a model for many textbook illustrations of the planarian eye is the work of Hesse (1897), whose description of the visual end organ was subsequently modified by Taliaferro (1920). The latter investigator also posed the possibility of analogizing, and possibly homologizing, the retinulae of the turbellarian eye with the receptors of the vertebrate eye. Analogies between vertebrate and invertebrate eyes have been extended by Wolken (1958), who deemed the fine structure of the sensory cells of the planarian eye to resemble the outermost portions of vertebrate retinal components. Since preliminary studies by the present author tended to contradict this view, an electron microscopic examination of the planarian eye was deemed useful not only from the point of view of clarifying its morphology, but also from the standpoint of providing evidence as to a possible analogy of platyhelminth and chordate eyes.

MATERIALS AND METHODS

Specimens of *Dugesia tigrina* were cut in two transversely at a level just behind the auricles, and the anterior portions were fixed immediately in a solution of 1% osmium tetroxide buffered at a pH of approximately 7.2 with a veronal acetate buffer. Following a period of fixation ranging from 20 minutes to 2 hours, the specimens were washed in distilled water, dehydrated in ethanol, and embedded in a mixture of 30% methyl methacrylate and 70% N-butyl methacrylate.

Sections, cut on an International Minot rotary microtome set to cut at 0.025 μ, were mounted on grids previously coated with a thin collodion membrane. The electron microscope used was an RCA model EMU 2.

Material for study with the light microscope was fixed in Bouin's fixative, dehydrated in ethanol, and embedded in paraffin. Sections were cut at 6 μ and stained with Heidenhain's iron hematoxylin.

DESCRIPTION

Observations with the light microscope show that the portion of the retinula (R, Fig. 1) found within the pigment cup exhibits a different capacity for staining

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[1] This study was supported in part by a grant from the Research Committee of the University of Wisconsin with funds from the Wisconsin Alumni Research Foundation. I am indebted to Professor Paul Kaesberg of the Department of Biochemistry, University of Wisconsin, for allowing me to use his electron microscope facilities.

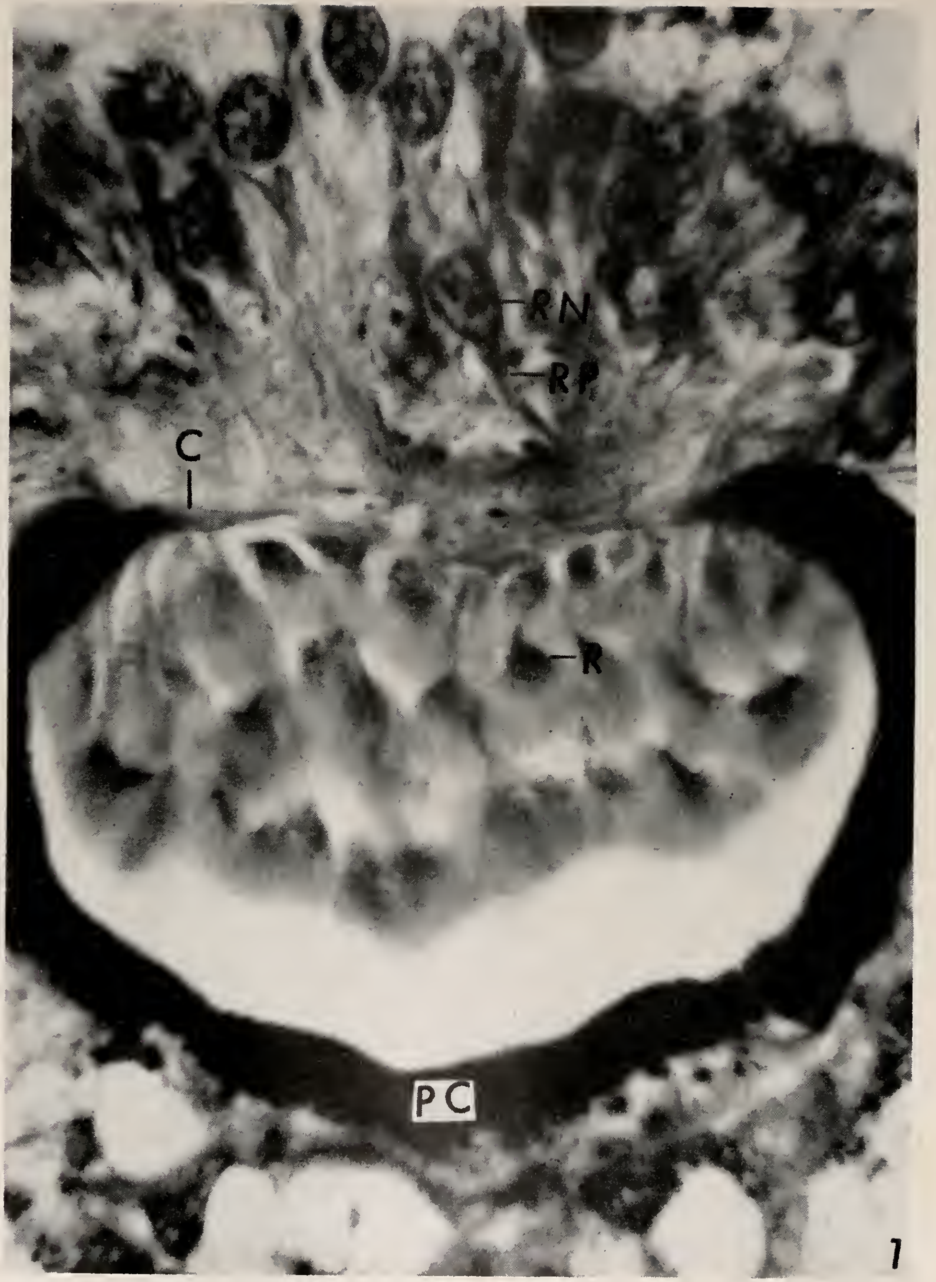


FIGURE 1. Light micrograph of an approximately frontal section, showing the pigment cup (PC), covering of the aperture of the pigment cup (C), retinula (R), nucleus of retinula (RN), and process of retinula (RP). The space between the most distal portions of the retinulae and the inner surface of the pigment cup is probably an artifact induced by fixation. × 1870.

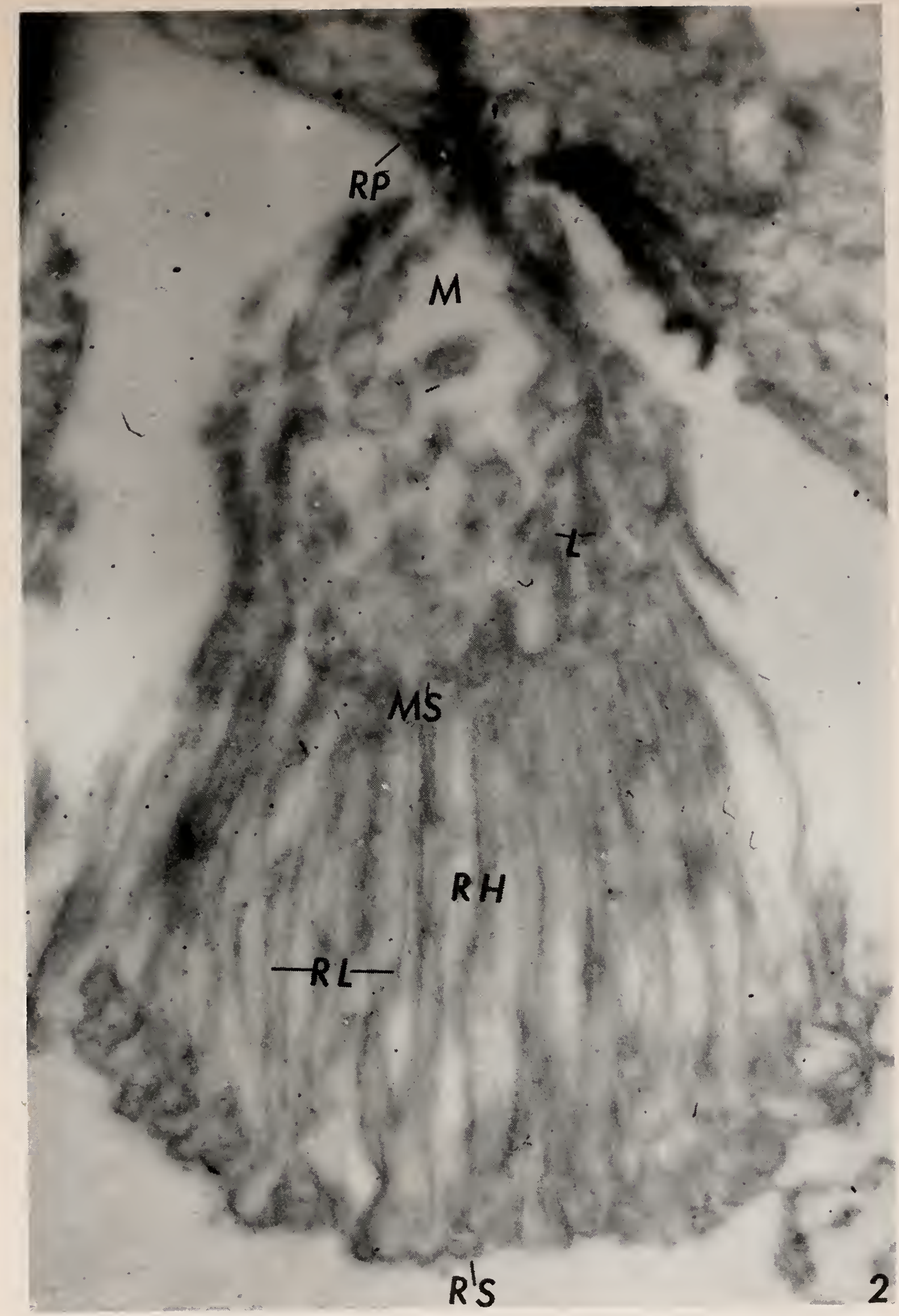


FIGURE 2. Electron micrograph of an approximately longitudinal section through a retinula, showing the retinular process (RP), the lamellae (L) of the "middle region" (M), and the bulb-like swellings (RS) at the distal portions of the lamellae (RL) of the rhabdome (RH). A delicate membrane (MS) separates the middle and rhabdome regions of the retinula. × 20,900.

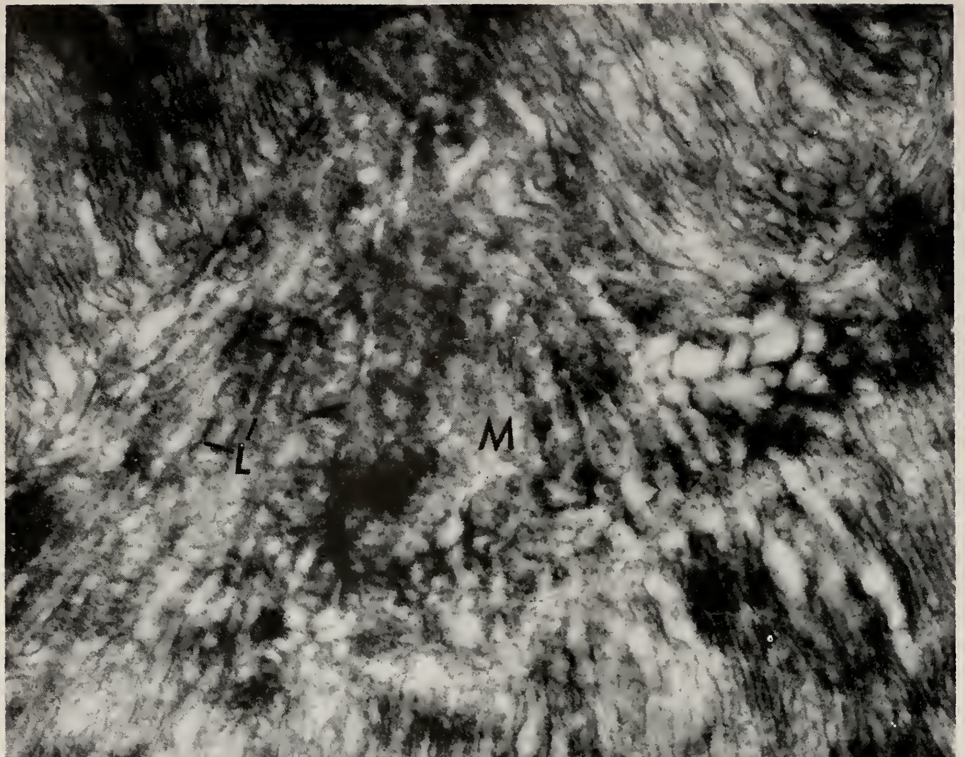
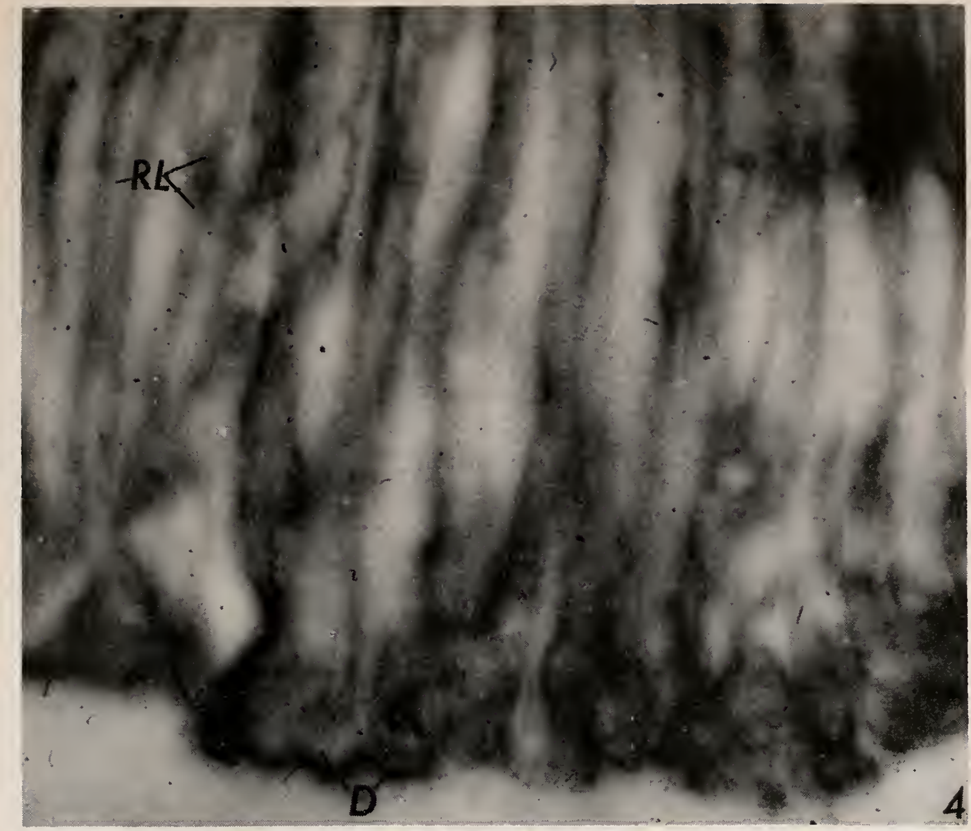


FIGURE 3. Electron micrograph of an approximately longitudinal section through a retinula, showing the lamellae (L) of the "middle region" (M), and the lamellae (RL) of the rhabdome (RH). Portions of rhabdomes of other retinulae are evident in the upper corners of the figure. × 19,000.



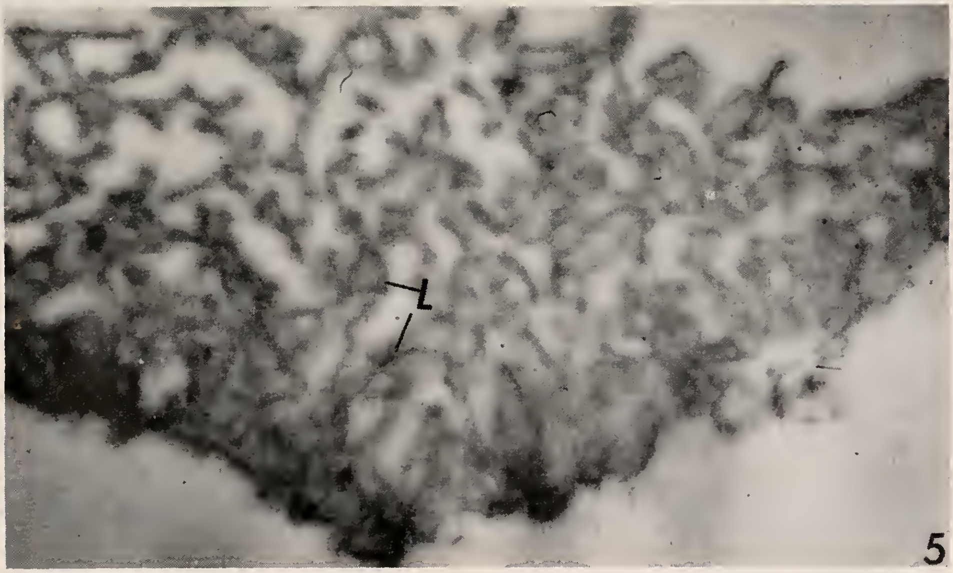


FIGURE 4. Distal portion of rhabdome of retinula seen in Figure 2, here shown at higher magnification, indicating the lamellae (D) in the distal swellings of rhabdome lamellae (RL). × 35,000.

FIGURE 5. Approximately transverse section through a portion of the "middle region" of a retinula, showing the lamellae (L) of that region. × 30,000.

with Heidenhain's iron hematoxylin from the rest of the retinula. The most distal portion of the sensory cell, which has been called the rhabdome by Taliaferro (1920), retains little of the stain and exhibits no internal structure save what has been termed a "rod border" (Hesse, 1897) or longitudinally oriented "striae" (Taliaferro, 1920). Proximal to the rhabdome of the retinula is a "middle region" staining intensely with iron hematoxylin, which continues proximally as a narrow process of the retinula. This latter process remains moderately intensely stained.

The electron microscope studies reveal that the whole of the rhabdome of the visual sensory cell consists of longitudinally oriented lamellae (RL, Figs. 2–4). These lamellae range in length from 3.18 μ to 4.20 μ, and in thickness from 300 Å to 490 Å. The distal portions of some lamellae are further differentiated into bulb-like swellings (RS, Fig. 2) bearing smaller lamellar structures with a maximum length of 0.44 μ that vary in thickness from 140 Å to 230 Å (D, Fig. 4).

A delicate membrane appears to delimit the "middle" from the rhabdome of the retinula (MS, Fig. 2). The sole discrete structures within the "middle region" (M, Figs. 2 and 3) are fiber-like lamellae (L, Figs. 2, 3, and 5). These lamellae vary in thickness from 160 Å to 610 Å, and appear in several instances to extend into the rhabdome.

Discussion

It has been suggested that the rhabdome of the retinula is its photosensitive region (Hesse, 1897; Taliaferro, 1920). In the visual biochemical reactions which presumably occur in the rhabdome, a lamellar arrangement would present a large surface area, particularly in a direction perpendicular to the longitudinal axes of the rhabdome vesicles. However, Taliaferro, in his experiments involving the locomotor responses of *Planaria* to light, has concluded (p. 113) that, "Light must strike a given rhabdome parallel with its longitudinal axis in order to cause stimulation of the rhabdome." But this direction is precisely the one which would least directly strike the longitudinal lamellar surfaces of the rhabdome. On the other hand, it is the most favorable direction by which light may reach the bulb-like swellings at the distal portions of the rhabdomal lamellae. The differentiated areas at the distal portions of the rhabdomal lamellae may be significant as possible sites of visual biochemical reaction.

The existence of a "middle region" as a distinct structure in the retinula of the planarian eye was not recognized by Hesse (1897), who figured in its place a fibrillar structure which was continuous distally with the "rod border" and proximally with fibers running to the cell body. In the preceding year Jänichen's (1896) description of the planarian eye showed a middle region in the retinula. In 1920, Taliaferro emphasized the importance of the "middle region," stating (p. 105), "that possibly this region serves as a crude lens to concentrate the rays of light upon the sensitive rhabdome and that photic stimulation depends upon this." Another possibility, however, is that these lamellae may transmit the impulse propagated by light-stimulation of the rhabdomal constituents. The latter explanation finds some support in the fact that some lamellae can be seen to continue into the rhabdome region.

As for the possibility of analogizing or homologizing the turbellarian and chordate eyes, the most that can be said is that certain similarities do exist between the two types. Both kinds of eye are of the inverse type, having the most distal portions of the receptors directed away from the opening of an eye partially lined with opaque pigment. Both kinds have visual receptors, each consisting of at least three distinct portions. The rods and cones of the vertebrate eye lie next to one another in approximately the same plane, forming a relatively flat retina lining part of the interior of the eye. In contrast, although the retinulae of the eye of *Dugesia tigrina* tend to be so oriented that their longitudinal axes are approximately normal to the nearest portion of the inner surface of the pigment cup, this orientation is far from consistent. In addition, the retinulae do not seem to lie in a single plane, but rather are distributed throughout the eye at varying distances from the inner surface of the pigment cup. Further, the fine structure of the planarian retinula differs markedly from that of the vertebrate rod or cone as described by several investigators (De Robertis and Lasansky, 1958; Sjöstrand, 1953; Wolken, 1958). Especially significant is the difference in orientation of the lamellar component, which is transverse in vertebrate receptors, but longitudinal in the planarian retinulae.

SUMMARY

1. Anterior portions of *Dugesia tigrina* were prepared for electron microscopic examination by fixing in osmium tetroxide buffered at pH 7.2, embedding in methacrylate polymer, and sectioning at 0.025 μ.

2. The rhabdome of the retinula is composed of longitudinally oriented lamellae whose distal portions are differentiated into bulb-like swellings bearing smaller lamellae. The "middle region" of the retinula contains lamellae that appear, in some instances, to extend into the rhabdome.

3. Little analogy can be drawn between the fine structure of the retinula of the planarian eye and that of the rods and cones of the vertebrate eye.

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STUDIES OF EARLY CLEAVAGE IN THE SURF CLAM, SPISULA SOLIDISSIMA, USING METHYLENE BLUE AND TOLUIDINE BLUE AS VITAL STAINS[1]

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In 1942 Iida described the behavior of certain particles stainable with neutral red in living eggs of a Japanese sea urchin. He found the particles to be one-half to one micron in diameter, to gather specifically into the regions of the asters during cleavage and to be distributed to the daughter cells by the mitotic spindle. In addition, he noted that the particles often moved very rapidly (several microns per second), but that their movement, especially after the establishment of the asters, was restricted to a direction radial to the centrosome. He also noted that particles would move both toward and away from the centrosome, and that of two neighboring particles, one might move and the other not. He thought that the particles were attached to astral rays and that the particle movement indicated intermittent growth (with periods of retraction) of the astral fibers. He compared both the form and activity of the aster to those of a radiolarian, centrosome representing the body of the radiolarian and astral fibers its filopodia.

Independently, Pasteels (1955, 1958), Pasteels and Mulnard (1957), Dalcq (1957) and Mulnard (1958) described particles stainable with basic dyes such as toluidine blue in living eggs of molluscs, annelids, echinoderms and ascidians, which at cleavage showed a behavior similar to, if not identical with, that first described by Iida (1942). Dalcq (1954) showed that rat eggs probably possess similar particles. The more recent work was considerably more complete and traced the origin and distribution of the particles from fertilization to later cleavage stages.

In addition, histochemical work indicated that acid phosphatase and acid mucopolysaccharides (the latter having been suggested by the fact that the particles stain metachromatically in the living egg) showed a distribution during cleavage similar to that shown by the particles in the living egg. Further, two types of particles were described, α-mitochondria not astrally located, appearing in the early fertilized egg, and β-mitochondria, appearing just prior to mitosis and showing the astral localization. Evidence was adduced to support the concept that the β-mitochondria were themselves not directly stainable, but became stained with the transfer of some substance to them from the α-mitochondria.

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[1] This work was partially supported by grants from the American Cancer Society and from the National Science Foundation, and was completed in part while the author was at the Department of Anatomy, College of Medicine, University of Illinois.

[2] We wish to dedicate this paper to the memory of Dr. Victor Schechter who was our first teacher in Biology and who contributed much to present knowledge of the properties of the egg of *Spisula solidissima.*

The work to be reported below was started in 1956 without knowledge of these antecedent results and took as its starting point, observations on vital staining of invertebrate eggs reported in Worley and Worley (1943) and Worley (1944a, 1944b). In this work, methylene blue was used as a vital stain and a number of molluscan and other invertebrate eggs were used as material. Certain bodies, called "Golgi" bodies by the above workers, were seen in the cytoplasm after staining, and an elaborate cycle of growth, fractionation and re-growth of these bodies during cleavage was described. This cycle was thought to be correlated to the synthesis and elaboration of lipid and protein yolk.

Our interest in this work stemmed from observations made in the course of electron microscope studies of developing eggs of the surf clam, *Spisula solidissima* (Rebhun, 1956a, 1956b). Cytoplasmic bodies considered to fulfill the criteria for yolk nuclei were there described and it was hypothesized that "heavy" protein yolk was manufactured in them, although the evidence was weak and mostly by analogy to observations and conclusions concerning yolk nuclei found in the classical literature (see, *e.g.*, Wilson, 1925). The yolk nuclei appeared to us to resemble the "Golgi" bodies of the Worleys (Worley and Worley, 1943), especially in the lamellibranch *Mytilus,* whose egg resembles that of *Spisula* in many respects. This resemblance, coupled with the suggestions of yolk synthesis claimed for each body, led us to the hypothesis that the two descriptions were, indeed, concerned with only one object (Rebhun, 1956b). This hypothesis, which led to the work described below, was, however, negated at our next opportunity to study living, stained eggs; *i.e.*, yolk nuclei are visible as refractile bodies in living eggs but never stain with methylene blue. During the course of these studies, however, we found that the particles stainable with methylene blue undergo the localization changes during cleavage described for neutral red particles in sea urchins by Iida (1942).

After this paper was accepted for publication, the author became aware of two papers by Kojima (1959a, 1959b) in which particles stainable with toluidine blue, neutral red, etc., were shown to exhibit the same behavior as those described below. In addition, Kojima showed the particles to be involved in the process of cell division. This work is discussed in a later section.

The following paper is an expansion of results already presented in preliminary reports (Rebhun, 1957, 1958).

MATERIALS AND METHODS

Oocytes were removed from ripe ovaries of the surf clam, *Spisula solidissima*, and washed following the procedure described in Allen (1953). They were then stained by either of two different procedures: that of Worley and Worley (1943), used throughout the major part of this work, and that of Pasteels (1955), used after we became aware of the latter's observations. In addition, some experiments were done with neutral red following Kojima (1959a).

In the method of Worley and Worley (1943), which we will call method I, eggs were stained by leaving them on a sea table at 19°–23° C. for ½ hour in a solution of 1 part methylene blue per 1,000,000 parts sea water, ½ hour in 1 part methylene blue per 500,000 parts sea water, 1 hour in 1 part methylene blue in 250,000 parts sea water, and finally, 1 hour in 1 part methylene blue in 125,000 parts sea water. Since there is variability in staining capacity in different batches of eggs (and with different lots of dye), the above schedule was somewhat varied in terms of staining time and final dye concentration used. However, a concentration of 1 part methylene blue per 125,000 parts of sea water was not exceeded. The eggs were then thoroughly washed by gentle centrifugation in fresh filtered sea water. The eggs of *Spisula* are very hardy (Schechter, 1941) and this long sojourn in dye solution does not appear to cause injury to the eggs. This may be judged by the fact that they cleave with the same frequency and at the same rate as control unstained eggs from the same batch, and that they develop for the same length of time on the sea table (namely, about three weeks), reaching the same final stage.

Eggs stained as above will be called "lightly" stained eggs. Their cytoplasm contains many small (about ¼ to ½ micron) particles, each heavily stained. If eggs are left in the highest concentrations of dye for 1 to 2 hours longer than described above, the particles appear to increase in size (but not number) until they are 1 to 2 microns in diameter, without, however, showing any dilution in color intensity. Such eggs will be called "heavily" stained eggs. Azure A and Azure B were also used in this procedure and yielded similar results, except that developmental anomalies were more frequent with the Azures than in unstained controls. These dyes give more intense staining, and concentrations of 1 part dye per 250,000 parts sea water were not exceeded with them.

The above method is obviously suited only to unfertilized eggs because of the extensive staining periods, and, therefore, for many purposes, the method of Pasteels (1955) was used. We will call this method II. Solutions of toluidine blue at concentrations of about 1 part dye per 100,000 parts sea water were used (this is about one-fifth the concentration used by Pasteels, 1955), and the eggs were allowed to remain in such solutions for from 2 to 5 minutes. The eggs were then removed and washed thoroughly by repeated centrifugation with fresh filtered sea water in a small hand centrifuge. Concentrations of toluidine blue of 1 or more parts dye in 50,000 parts sea water will stain the thin jelly coat and the vitelline membrane, reduce the fertilization percentage, often induce some of the eggs to develop parthenogenetically, and cause the appearance of angular and elongate particles in the cytoplasm which may be crystalline aggregates of dye. At the lower concentration of 1 part toluidine blue in 100,000 parts sea water, eggs stained when unfertilized will subsequently show no difference in fertilization percentage, cleavage rate and length of developmental period on the sea table, when compared to "sibling" controls. Similarly, eggs stained with the lower dye concentration at any time during the mitotic cycle compare closely with controls in the above characteristics.

In some experiments "mitochondrial" techniques were used. The Nadi reaction was used according to Ries (1937). Zinc-free Janus Green B (kindly given to us by Dr. D. P. Costello) solutions at a concentration of 1 part dye in 50,000 parts sea water were used to stain eggs at various times in the mitotic cycle. Finally, the NBT method of Nachlas *et al.* (1957) was tried. Although this latter technique did not yield consistent results as far as appearance of eggs from different batches is concerned, it did induce interesting anomalies in cleavage which will be reported at a later date.

Photomicrographs were taken through a Leitz Ortholux microscope with Micro-ibso attachment and Leica camera using Kodak Microfile film and D-19 developer. Over 2000 feet of time-lapse movies were made with Plus X and Tri-X 16 mm. movie film of fertilized, stained eggs at many periods from fertilization to fifth cleavage. The movie equipment used consisted of a Bolex movie camera and Samenco movie control box. The camera was supported above the microscope and was driven at rates of 1 frame per second, or 1 frame per 2 seconds by a relay actuated by the control. The light is on constantly with the Samenco control for speeds as rapid as the above. Films were analyzed visually by timing events from a screen.

In some experiments, stained eggs were centrifuged in a small high speed electric centrifuge, the speed of which was altered with a Variac. The centrifuge was calibrated with a stroboscopic lamp (Strobotac). Eggs were centrifuged against isotonic sucrose (0.95 molal) in Pyrex tubes (Kopac, 1955). Complete stratification could be obtained with 1½ to 4 minute spins at 8000 g (depending on the batch of eggs), but various regimes were tried on a given sample of eggs, *e.g.*, 1 minute at 2000 g, 1 minute at 5000 g, and 2 minutes at 8000 g. This was considered to lessen the chance of accidental trapping of particles, *e.g.*, above the nucleus. A more complete description of centrifugation techniques will be reported in the observations section.

Observations with several phase systems and the Baker interference microscope (using both shearing and double focus systems) were made on normal eggs at various times in the cleavage cycle. To help alleviate the halo and scattering effects due to refractile granules in the cytoplasm, Dr. Keith Ross and the author attempted to find an immersion medium (Ross, 1954) which would allow cleavage to proceed normally and which would match the average refractive index of the egg cytoplasm. We used bovine serum albumin, Armour fraction V in sea water, gum arabic in sea water, and a high molecular weight polyglucose, "Ficol," dissolved in sea water. Concentrated solutions were made and dialysed against sea water in a refrigerator overnight. Eggs were immersed in this medium (or dilutions thereof) on slides and were then observed.

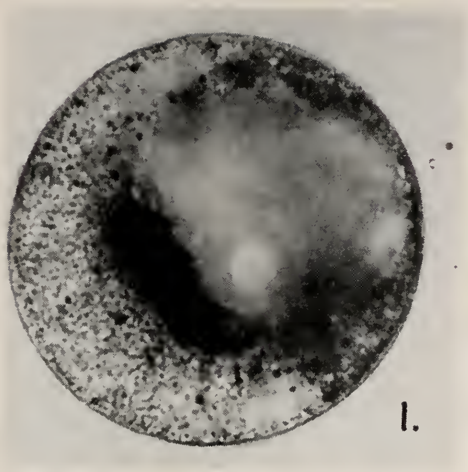
A complete report of our electron microscopic techniques will be made at a later date, although some of the results already obtained will be mentioned below.

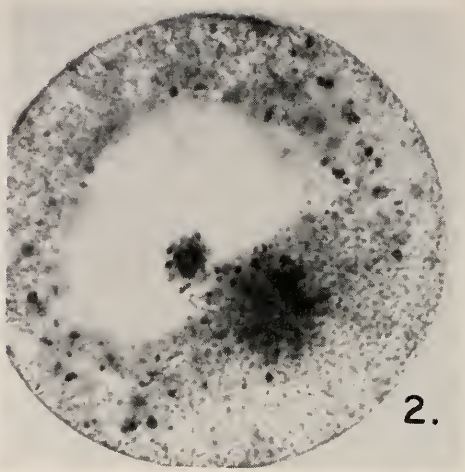
OBSERVATIONS

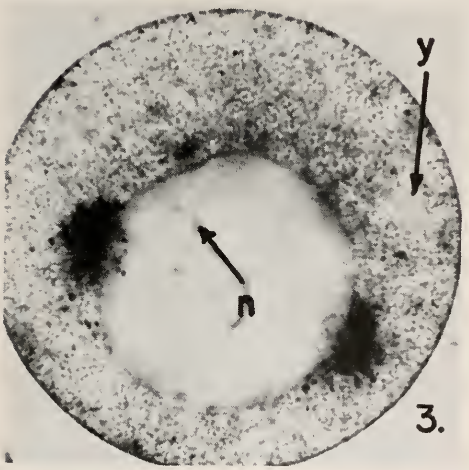
I. *Unfertilized eggs:*

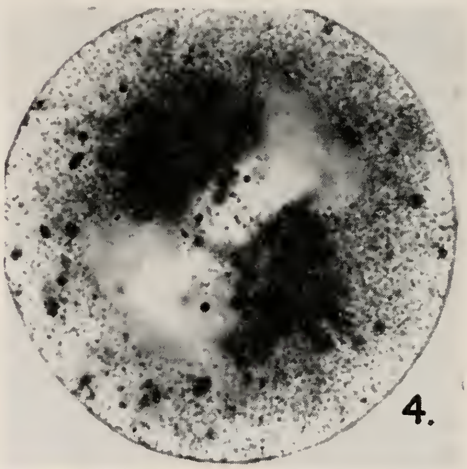
a. *Method I*

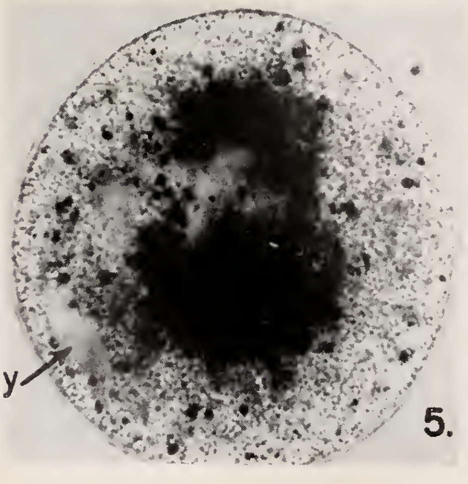
As mentioned above, small stained particles appear in eggs after several hours in solutions of methylene blue. With increased staining time the particles grow from about ¼ to ½ micron up to 1 to 2 microns in diameter. This does not appear to cause a decrease in the light absorption of the bound dye as might be expected if the dye were being diluted in a swelling vesicle. The particles appear dark whether the egg is lightly or heavily stained, and in fact, the larger particles appear darker. When eggs are removed from the staining solutions, by repeated washing in sea water, the particles do not increase further in size. Sim-

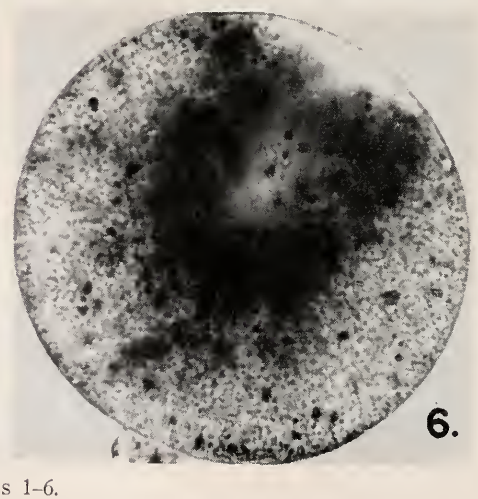












Figures 1–6.

ilarly, fertilization of eggs in staining solutions appears to prevent further increase in the size of the particles even though the dye is still present in the sea water.

Eggs left in staining solutions for 6 to 8 hours begin to show the formation of compound bodies with several particles aggregated into rows or around clear areas ("vacuoles") of cytoplasm 3 to 5 microns in diameter. Occasionally, very large spherical bodies up to 10 microns or more may be seen, which stain heavily with dye. We believe these compound bodies to be artifacts produced in the continued presence of dye. Yolk nuclei are visible in unstained and stained eggs (*e.g.*, see Figure 3), but, as mentioned in the introduction, never take up dye although a stained particle or two may appear within the interior of the spherical type of yolk nucleus (Rebhun, 1956b).

b. *Method II*

After short-period staining with toluidine blue, very small particles (¼ to ½ micron in diameter) appear in the egg and are uniformly distributed throughout the cytoplasm in most eggs. However, in some eggs, there appears to be a greater localization in the cytoplasmic region nearer to the nucleus. The particles take a reddish hue; *i.e.*, they stain metachromatically. The outermost layer of cytoplasm (about 1 to 2 microns thick) just beneath the vitelline membrane contains a set of particles which are somewhat elongated and are about 1 micron in over-all length although much variability in size exists. With the staining regime of method II, these cortical granules stain light blue, that is, non-metachromatically (in methylene blue solutions the granules stain light green). The two types of stained particles are thus easily distinguished by size, color, and location.

II. *Fertilized eggs:*

a. *General*

Particles in lightly stained and heavily stained eggs (method I) (taken out of dye solutions before compound bodies formed) and those stained by method II showed approximately the same behavior during fertilization and cleavage. However, until the stage of syngamy there is much more "diffuse" coloration (metachromatic) with method II than method I, which tends to obscure the events in the early stages. Most of the photomicrographs and movies, therefore, were taken

FIGURE 1. A lightly centrifuged egg with methylene blue stained particles accumulating just centrifugal to the nucleus.

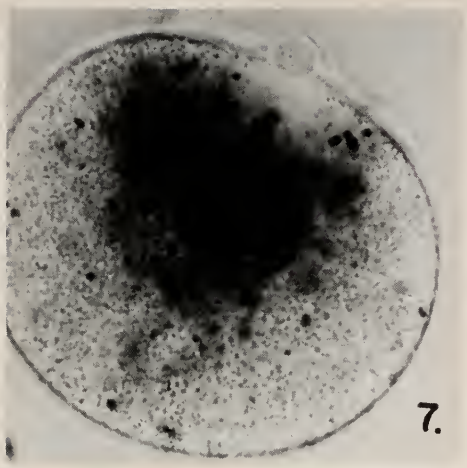
FIGURE 2. A parthogenetically activated, centrifuged egg. The aster is expanding into the nucleus.

FIGURE 3. A fertilized methylene blue stained egg. Many of the particles are gathered about the egg centrosomes, and astral rays are just beginning to indent the nuclear surface. About 8 minutes after fertilization. N = nucleolus, y = yolk nucleus.

FIGURE 4. At about 13 minutes after fertilization the astral rays have bridged the nucleoplasm and the spindle is being established. Note the masses of nucleoplasm at either side of the spindle.

FIGURE 5. At about 18 minutes the spindle is complete and begins its peripheral migration. The "excess" nucleoplasm has become intermingled with the cytoplasm.

FIGURE 6. At about 25 minutes the peripheral position of the spindle is established. Note the clear peripheral area. This is reminiscent of that seen later for first and second cleavage spindles.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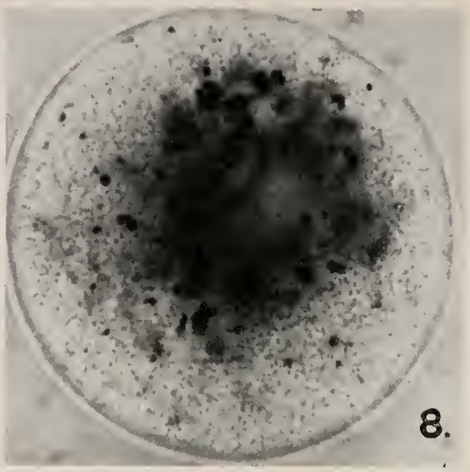
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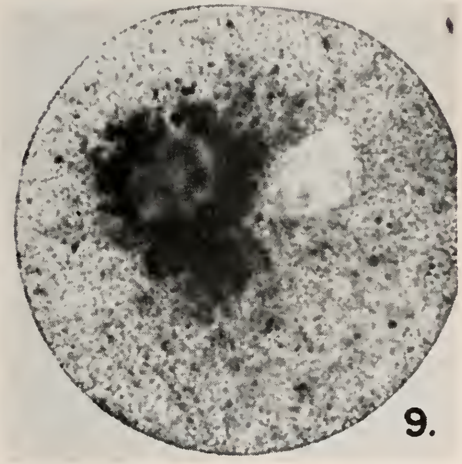
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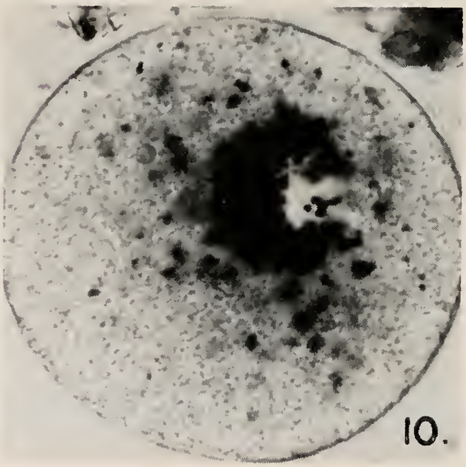
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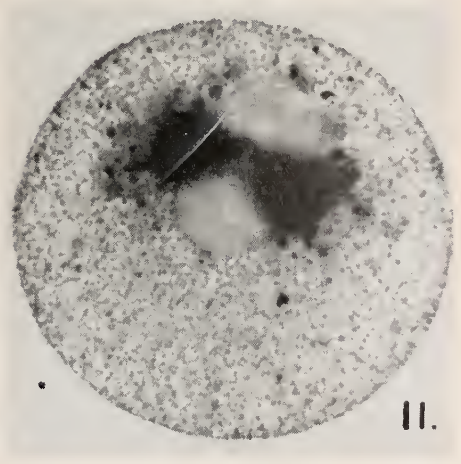
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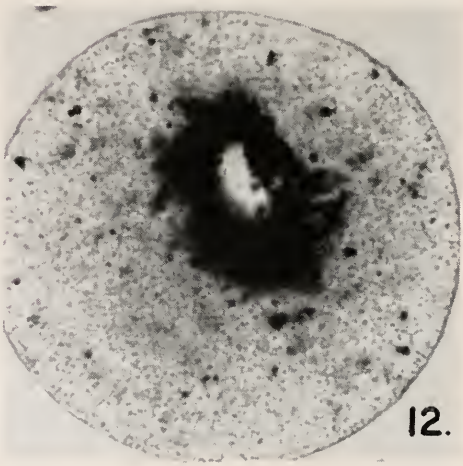
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FIGURES 7–12.

with heavily stained eggs which lack this "diffuse" coloration. Unfortunately, these eggs appear to be more sensitive to the light (or heat) of the microscope lamp than lightly stained eggs or unstained controls (despite heat filters and water cells) and movies of them cannot usually be taken for longer than 15 minutes. However, heavily stained eggs develop perfectly well on the sea table so that slides can be made as needed from a batch fertilized at one time.

b. *Formation of the first polar body spindle*

At about 6 to 8 minutes after fertilization, asters may be seen in the cytoplasm of the eggs, adjacent to the nuclear membrane: these may appear facing each other across the germinal vesicle or may be nearer to each other along the nuclear surface. A minute or two later the nuclear surface appears to soften, the nucleus actually enlarging (see Figure 3). The asters also begin to enlarge and the rod-like astral fibers facing the nucleus appear to push their way into the nucleus through its softened surface (Fig. 4). They elongate at the rate of about 1 micron every 2 to 3 seconds, occasionally pushing refractile (not necessarily stained) granules ahead of them from the cytoplasm into the nucleus. The cytoplasmic granules accompanying the nuclear invasion by the asters form a bridge of cytoplasmic material between the two asters (Fig. 4). In some eggs the stained particles begin to show a specific movement toward the astral centers. This is developed to a variable extent in different eggs at this time but is often quite definite. As the spindle (first polar body spindle) forms between the asters, the particles migrate more and more into the astral regions. The spindle elongates after its formation and at about 15 minutes after fertilization begins to move from the center of the egg toward the surface where the first polar body is given off (Figs. 5, 6).

When the spindle first forms, two large clear areas of nucleoplasm can be seen on either side of it (Fig. 4). They may be unequal in size, depending on the positions of the asters just before the nuclear membrane disappears. When the spindle begins its peripheral migration this nucleoplasm becomes increasingly mingled with the cytoplasm and finally can no longer be distinguished as a separate

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FIGURE 7. The first polar body is formed by 31 minutes and the egg still has its oblate spheroid shape. Most of the particles are aggregated about the spindle or are, at least, in the animal hemisphere.

FIGURE 8. An optical section of the first polar body spindle perpendicular to its axis. The particles are distributed relatively uniformly. Note that many of them are aggregated into larger masses. Although difficult to photograph, these masses appear to be aggregates of smaller particles when studied visually.

FIGURE 9. The male pronucleus has not completely rounded out yet but is near the female pronucleus. About 50 minutes after fertilization.

FIGURE 10. A view along the line connecting the pronuclear centers reveals the particles lying approximately in a plane tangent to the two pronuclei at their point of contact (see Figure 11). The group of particles may be arranged in a crescent as here, or may form a complete ring. Fifty-three minutes after fertilization.

FIGURE 11. Looking at the pronuclei in a direction perpendicular to that of Figure 10 reveals the as yet intact pronuclei and the ring of particles in the plane tangent to the pronuclei. Same time as Figure 11.

FIGURE 12. The ring of particles begins to separate into two groups which will outline the asters of first cleavage. This process begins between 53 and 54 minutes after fertilization at 21° C.

entity (Fig. 5). Although some of the nuclear material may contribute to the spindle, the majority of it can be seen to mix with the cytoplasm.

From the time the asters first become visible, some of the stained particles migrate toward them (always excluding a zone about 4 to 5 microns in diameter centered on the centriole). This migration increases in intensity so that by the time the first polar body is given off at about 30 minutes, most of the particles are at least in the animal hemisphere, if not directly applied to the central aster and spindle (Figs. 6, 7). Some, but not all, of these particles move with great rapidity (several microns per second) and may appear to shoot quickly into the aster in time-lapse movies.

Many of the particles aggregate into what appear to be grape-like masses oriented radially along the astral rays (Figs. 6–8). This does not appear to be merely due to mechanical jostling since such aggregates may be seen in areas devoid of astral rays. When the spindle is first formed the stained particles outline each aster about equally. However, after the peripheral location of the spindle is established, the particles about the peripheral aster migrate to the sides of the spindle so that it is now outlined by a cup-shaped set of particles (Figs. 5 and 6). An optical section of the spindle and particles, perpendicular to the axis of the spindle, appears in Figure 8.

c. *Formation of first and second polar bodies*

At about 27 minutes after fertilization the egg begins to elongate in a direction at right angles to the animal-vegetal axis so as to become an approximate oblate spheroid (Fig. 7). The first polar body is given off at about 30 minutes (at 21° C.) and is accompanied by what appears to be a shortening, and a movement, of the spindle partly into the polar body (see Conklin, 1902, for a similar phenomenon in *Crepidula*). One or two stained particles may move into the polar body. The egg then rounds up.

Ten minutes later the second polar body is formed after a change in egg shape similar to that which occurred in first polar body formation. Throughout this period more and more of the laggard stained particles have been moving towards the spindle area and by now very few can be found elsewhere in the egg. A tight, organized mass of these particles exists around the second polar body spindle and central aster, and within this mass one can see, by careful focussing, the groups of particles forming grape-like masses.

Again, it must be emphasized that these observations can be made in eggs stained by method I or method II, although they are easier to make using method I because of the "diffuse" stain with method II.

d. *Formation of the pronuclei and their subsequent migrations*

A few minutes after second polar body formation the female pronucleus can be seen with the attendant mass of stained particles. These may completely surround it or may be gathered at one pole (pointing essentially toward the egg center). Whatever the initial distribution, however, ultimately the particles gather at the central pole of the female pronucleus.

The earliest stages of male pronucleus formation have not been seen but at about the time that the female pronucleus becomes visible, the male pronucleus is already present and at this time appears as a clear body smaller than the female pronucleus and shaped like a short, very thick exclamation mark pointing toward the egg interior (Fig. 9). At the "period" of the exclamation mark is the center of the sperm aster which soon radiates throughout the cell. The male pronucleus increases in size and becomes spherical and about the same size as the female pronucleus.

The pronuclei have moved toward each other and by now the stained particles form a ring or crescent on the side of the female pronucleus facing the male pronucleus. The two pronuclei soon come into contact. A line drawn through their centers runs parallel to the animal-vegetal axis in the most frequent cases. The ring of particles is bisected by a plane which is tangent to the pronuclei (and thus perpendicular to the animal-vegetal axis). For views along and perpendicular to the animal-vegetal axis, see Figures 10 and 11, respectively.

It will be noticed in the above description that the particles stayed with the female pronucleus and did not move toward the male aster. This has been seen invariably in these eggs with both staining methods. However, in polyspermic eggs some particles may be seen to move into the sperm asters, usually with great rapidity (about a micron per second or so), although the bulk of particles remain with the female pronucleus.

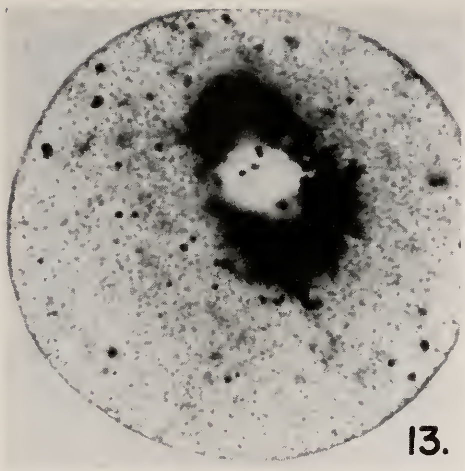
Preliminary studies have been made on parthenogenetic eggs stained with methylene blue and stimulated with KCl (see Allen, 1953). The events up to second polar body formation appear to be identical to those in the normal fertilized egg. However, a female pronucleus does not form in such eggs, although several small clear vesicles do, and, although observation of their relation to the particles is difficult, it is likely that the particles associate randomly with the vesicles. It would appear that the vesicles are similar to the karyomeres which form just prior to blastomere nucleus formation in first cleavage (see below). Although vesicle and chromosome counts were not made, it appears likely that each chromosome forms a karyomere and that, in parthenogenetic eggs, fusion of the karyomere to form a nucleus (pronucleus) does not occur.

e. *Formation of the first cleavage spindle*

The two pronuclei are now in contact and the nuclear membranes soon break down, liberating the nuclear contents without forming a true fusion nucleus. Just prior to this, the ring of stained particles divides into two half rings when seen along the animal-vegetal axis, and the half rings soon round up into partial spheres surrounding each centrosome. (See Figures 12 and 13 for two views of the same egg, about 20 seconds apart and just before nuclear membrane breakdown.) In compressed eggs it can again be seen that the particles are excluded from a region 4 to 5 microns in diameter around the centriole.

When viewed in a direction perpendicular to the animal-vegetal axis the mass of stained particles appears as a bar of material tangent to the two pronuclei (Fig. 11). This soon separates into two masses, corresponding to the division of the ring seen from the perpendicular direction.

The spindle forms in the center of the egg and remains there for about 3 minutes (Fig. 14). It is then translated along its axis to the periphery of the egg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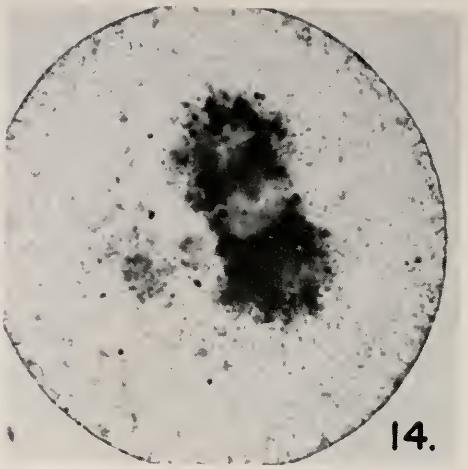
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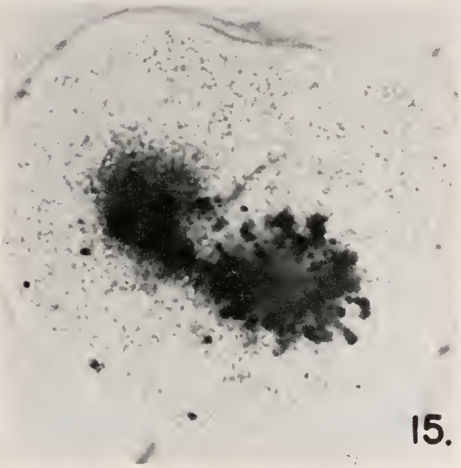
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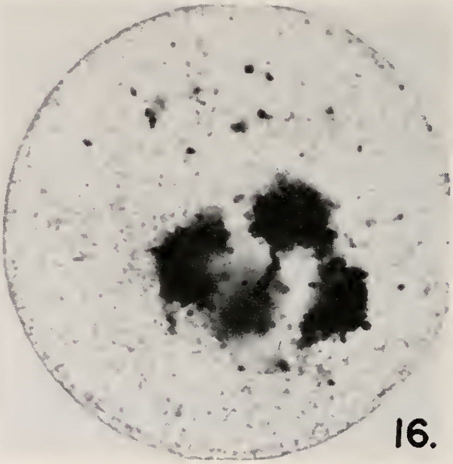
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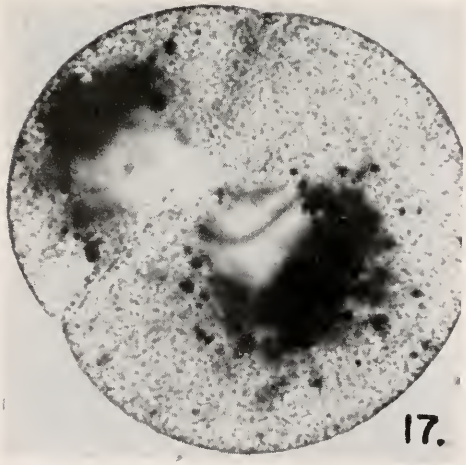
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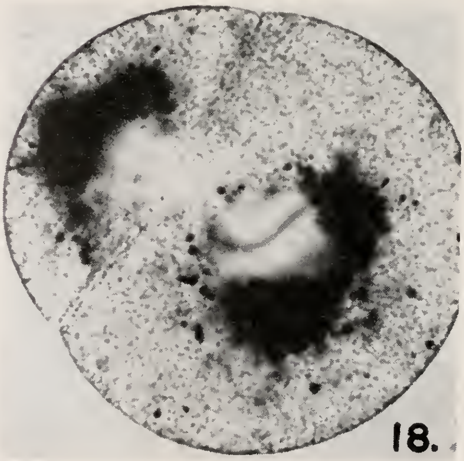
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FIGURES 13–18.

just prior to cleavage. In time-lapse movies where a frame is taken each 2 seconds, this movement looks very rapid since the whole process takes only about 45 seconds in life. The spindle is now about 30 microns in length (the egg is about 60 microns in diameter) and its axis is perpendicular to the animal-vegetal axis (Fig. 15). In the majority of eggs it appears (with accurate counts not taken) that the number of stained particles about the peripheral aster is smaller than that around the central one (for a similar phenomenon in second cleavage, see Figure 23). Although the maneuvers of the stained particles in polyspermy have not yet been studied in detail, the end result has been seen many times. The stained particles appear to divide among the asters present in approximately equal numbers so that the multipolar spindles are neatly outlined at their astral apices (Fig. 16).

f. *Spindle rocking and first cleavage*

A detailed examination of the peripheral aster at this stage reveals that a cone of clear cytoplasm free of granules of any type exists from the centrosome to the egg surface (a distance of about 2 to 3 microns) (see Figure 23 for the same phenomena in second cleavage). The stained particles surround the asters, being excluded from the peripheral area just mentioned, the spindle itself and the centrosome.

Almost immediately after the spindle translates to the periphery it begins a peculiar, regular rocking motion. Although this was first seen in time-lapse movies of stained eggs, it may be seen as well in unstained eggs by visual observation. The half-period is about 30 seconds and from 4 to 8 half-periods are completed before the motion stops. The motion is one in which the central aster remains fixed and the peripheral one moves through an arc of about 30 degrees. By close observation of the clear cone between the peripheral aster and the surface, it can be seen that this clear region moves with the spindle and therefore appears to slide to and fro beneath the cell surface (Figures 20 to 22 show the rocking phenomena for second cleavage).

The movement stops just before spindle and cell elongation occurs. The cell elongates now, in a direction parallel to the spindle axis, and so forms a prolate spheroid with the spindle on its axis. Furrow formation starts at about this time and usually advances from the region of the polar bodies (animal pole) first. The plane of the furrow bisects the axis of the spindle and because of the peripheral location of the spindle, two unequal blastomeres are formed, the large CD and small AB blastomere. The stained particles outline the asters beautifully and, as

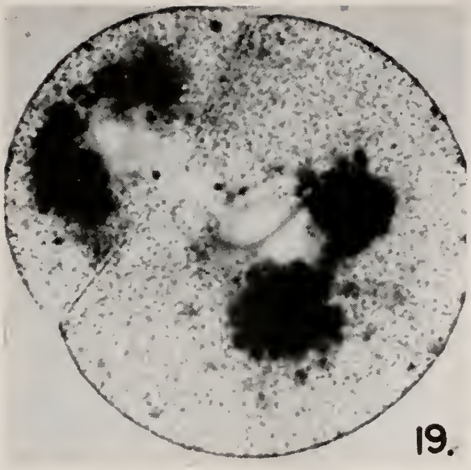
FIGURE 13. This view is of the same egg as in Figure 12, but taken approximately 20 seconds after it, and shows the beginning of the formation of the central concentration of the methylene blue stained particles.

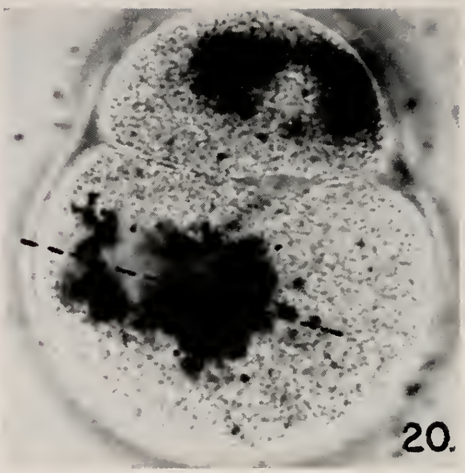
FIGURE 14. The asters are well formed and the spindle now begins to elongate. Note the similarity of Figure 14 with Figure 4.

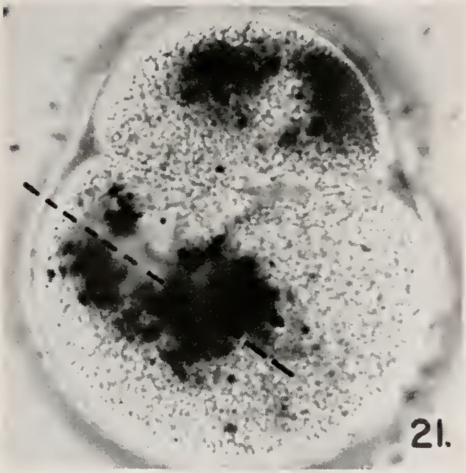
FIGURE 15. At about 60 minutes the first cleavage furrow starts to form, first at the animal pole. The egg has elongated in the direction parallel to the axis of the spindle. Note the radial arrangement of the clumps of particles in the aster and the "hollow" center it possesses.

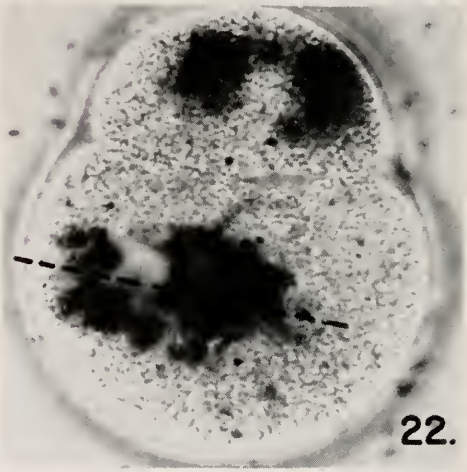
FIGURE 16. Four groups of astrosomes (one slightly out of focus) indicate the ends of a tetrapolar spindle in this polyspermic egg.

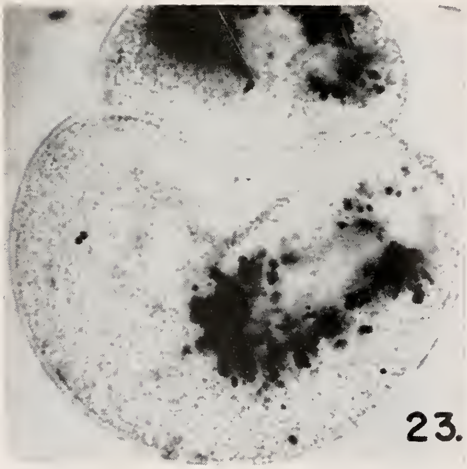
FIGURES 17–18. See Figure 19.

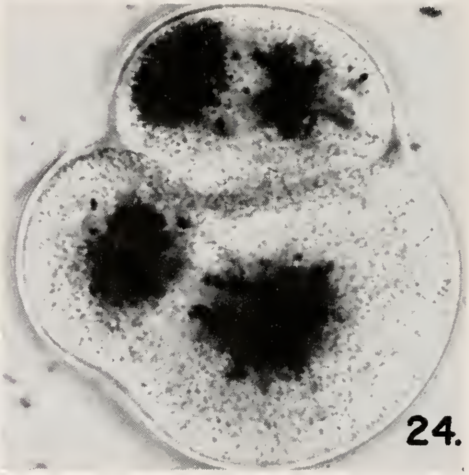












Figures 19–24.

can be seen from Figure 15, almost all have localized in the asters near the centrosomes at this time.

The blastomeres round up again after the cleavage furrow has gone through. The polar bodies remain at the animal pole, beneath the vitelline membrane.

In abnormal cases arising either in eggs which have been stained by method I long enough to form compound bodies or in eggs which have been damaged by prolonged exposure to the microscope lamp, the rocking motion may continue longer (12–20 half-periods) and may attain angles up to 45°. In these cases the motion then gradually subsides with no cleavage following. On the other hand, perfectly good cleavage can occur without spindle translation to the periphery or spindle rocking. In these cases the spindle remains central in the egg and cleavage yields two equal sized blastomeres. Such cases are rare, however, and are probably anomalies in terms of later development.

In addition to the above observations, fertilized eggs previously untreated with dye were stained by method II at first cleavage. In all cases, we observed a pattern similar to that seen in eggs stained before fertilization: that is, an astral location of the metachromatic particles. In some batches of eggs, there were few, if any, discrete stained particles anywhere else in the cell. However, in most other batches of eggs, many stained particles were seen scattered throughout the cytoplasm (appearing on casual observation as a "diffuse" stain), although a heavier astral concentration always existed. The stain in the astral regions was always light compared to that which would be seen at this time in eggs which had been stained before or just after fertilization, so that eggs often had to be compressed to see astral localization.

The further maneuvers of particles stained at this late date are identical in nature to those stained before fertilization.

g. *Interphase and prophase of second cleavage division*

Nuclear reconstitution takes place by the formation and fusion of small karyomeres ("chromosomal vesicles") as can be seen most clearly from electron micrographs. This same process occurs in *Chaetopterus*, *Arbacia* (Gross *et al.*, 1958) and *Fundulus* (Richards, 1917) and is probably of quite general occurrence in molluscs (Raven, 1958). The stained particles begin to gather at one pole of the blastomere nucleus, primarily that facing the periphery, although sometimes a little to one side. The particles are rarely found on the surface of the central hemisphere of the blastomere nucleus (Fig. 17). After formation of the cap of stained particles, one sees remarkable movements in some of them in time-lapse

FIGURE 19. Figures 17 to 19 are taken of the same egg about 20 seconds apart and indicate (a) the peripheral location of the astrosomes on the blastomere nuclei and (b) the separation of the particles into two groups just prior to nuclear membrane breakdown. Unfortunately, a piece of lint traverses the image of the nucleus in the CD blastomere.

FIGURES 20–22. Microphotographs of the same egg taken about 20 seconds apart. This shows the 30° excursion of the spindle in a half rotation. Note the fixed central aster.

FIGURE 23. The oscillation in this egg has stopped and the pre-cleavage elongation has occurred. Note the peripheral cone of clear cytoplasm, the radial arrangement of the astrosomes and the smaller number of particles about the peripheral aster.

FIGURE 24. The cleavage furrow is practically completed at about 75 minutes in the CD blastomere. The AB blastomere lags by about 1 minute.

movies or by direct visual use of the light microscope. They may move very rapidly (1 to 2 microns per second) in a radial direction, shuttling back and forth in straight lines between the nucleus and the periphery. In time-lapse movies they may appear to jump discontinuously.

This movement subsides before the next division, which occurs 15 minutes after first cleavage. The prelude to this cleavage is a division of the mass of stained particles into two masses which then appear to slide over the intact nuclear surface, ultimately coming to lie at opposite poles of the nucleus (Figures 17 to 19 show this process in one egg: the pictures are taken 20 seconds apart). This undoubtedly corresponds to prophase of the second division, but the nuclear events cannot be seen easily enough to identify early stages by chromosome morphology. The division of the mass of stained particles into two sub-masses occurs about 1 minute earlier in the large CD blastomere than in the small AB blastomere, which correlates with the 1-minute difference in cleavage times (Allen, 1953).

The particles forming the two masses are arranged so as to indicate the poles of the second cleavage spindle. It can then be seen that the axis of this spindle in each blastomere makes an angle of 90° with that of the spindle of first cleavage. On the other hand, the axes of the spindles of the two blastomeres in some, but definitely not all eggs, are tipped at about 30° to each other as can be seen when looking along a line parallel to the axis of the first cleavage spindle (see Conklin, 1902, for a similar observation in *Crepidula*).

h. *Second cleavage*

Second cleavage in the AB blastomere is easily described and presents no unusual aspects. The two groups of stained particles outline the asters and show the same characteristics described for first cleavage asters, *i.e.,* aggregation of particles into the aster, movement of particles in a direction radial to the astral center, etc. (Fig. 19).

The spindle for the CD blastomere forms approximately in the center of the blastomere. It then shows a sudden movement to the periphery and toward the AB blastomere (which has not yet divided). The movement takes about 45 seconds and results in the spindle axis, in its new position, making an angle of about 30° with the axis of the spindle in the position in which it first formed. Except for this tipping, the motion is reminiscent of the spindle translation seen in first cleavage. The resemblance is further strengthened by the occurrence of a rocking motion on the part of this spindle of about the same period, amplitude, and form as in first cleavage (Figures 20 to 22 show a period in the rocking motion, the photographs having been taken about 20 seconds apart). By the same form is meant the fact that it is the peripheral aster which moves, the whole spindle rocking about the fixed central aster. Again in conformity with the events in first cleavage, a cone of clear cytoplasm exists between the peripheral aster and the egg surface, and the peripheral aster with its cone appears to slide to and fro under the surface (Fig. 23). Finally, the number of stained particles near the peripheral aster is smaller than that near the central aster (Fig. 23). It is thus clear that cleavage of the CD blastomere is very similar in course to that of the whole egg in first cleavage.

The rocking motion stops after 4 to 8 half-periods and in its final position the spindle is tipped at about 30° to the animal-vegetal axis, that is, it has returned to the position from which it started its rocking motion (Fig. 23). The cell elongates approximately parallel to the spindle and the cleavage furrow then begins to pinch in, as in first cleavage, from the animal pole first. The result is a large D blastomere and a small C blastomere (Fig. 24).

As in first cleavage, in some cases the spindle fails to migrate to the periphery. The resulting blastomeres are then of equal size. In such cases irregular rocking motions of the spindle sometimes occur.

Later cleavages have not been studied in detail but the same process of division of the stained particles into two masses, outlining of the asters and, finally, reconstitution of a single mass on the nuclear surface has been followed as far as fifth cleavage and there is no reason to suppose it stops here. In addition, in two sequences of a time-lapse movie of third cleavage, the spindle of the D blastomere (but not that of the others) was seen to form in the center of the cell, move to the periphery, and show the same spindle rocking movements as seen in the whole egg in first cleavage and the CD blastomere in second cleavage.

It should be pointed out that the number of particles stained appears to remain the same from the unfertilized egg on, so that they appear to be diluted in each blastomere in each division. This by no means implies that new particles do not arise in each division but simply that if such particles arise it is probably not through division of previously stained ones.

i. *Centrifuged eggs*

Staining experiments with centrifuged eggs were carried out primarily with toluidine blue, although neutral red (Kojima, 1959a) was used in some runs, the results being essentially identical. The eggs were generally stratified at 8000 g for periods of 1½ to 4 minutes depending upon the batch of eggs (clams from different populations behave quite differently with respect to stratification times). In addition, a limited number of eggs were partially stratified at 4380 g for 1 minute at approximately the first polar body stage and at first cleavage.

Four types of experiments were run: (1) eggs were stained before fertilization and then stratified at various periods through second cleavage; (2) eggs were fertilized, stained for several minutes starting at 5 minutes after fertilization and then stratified at various periods through second cleavage; (3) eggs were fertilized and then stained for from 2 to 5 minutes immediately before centrifugation at various times through second cleavage; (4) eggs were fertilized, stratified at various times through second cleavage and then stained, usually for not more than 3 minutes, to avoid major redistribution of particles before observation.

Before describing the results of the above experiments, several considerations must be mentioned. First, the stratified egg begins to redistribute quickly, so that observations must be rapid. Thus, large numbers of eggs must be surveyed under low power and estimations of the location of stain in "typical" cases made in a matter of minutes. Cases may then be selected for longer study at high power (oil immersion). Second, eggs destain rapidly if oxygen is excluded from the preparation as is inevitable when compressed eggs are used. Third, in many cases the vitelline membrane and jelly coat stain heavily with dye (metachromatically) so that it is very difficult to decide whether a "diffuse" stain is, indeed, intra- or extracytoplasmic. Fourth, the cortical granules stain blue and interfere with observations in the centrifugal portions of eggs (see below). Fifth, many refractive yolk granules in the centrifugal parts of stratified eggs place considerable restrictions on accurate observation in this zone. In assessing the results of the experiments, the reader must bear the above considerations in mind.

After germinal vesicle breakdown, the unstained, stratified *Spisula* egg shows five constant layers, with some overlap in the more centrifugal ones: most centripetally, a cap of lipid granules; next, a clear layer; then, a mitochondrial layer; a yolk layer; and, finally, most centrifugally, a layer of cortical granules (these do not break down on fertilization in the *Spisula* egg). In addition, pronuclei may be found immediately below the lipid cap after second polar body formation, and a spindle after syngamy, although the latter is by no means easy to see.

1) *Behavior of particles in eggs with a germinal vesicle (unfertilized eggs to eggs about 10 minutes after fertilization.)* The results of experiments on such eggs are the same whether staining is carried out after or before centrifugation. In all cases the majority of the metachromatic particles can be seen in a narrow layer centrifugal to the germinal vesicle (a layer identified as a mitochondrial layer with the electron microscope) (see Figure 1). Usually, a small number of the particles can be seen in the lipid cap and in the centrifugal yolk area. In about 2 to 3 per cent of the cases, many of the cortical granules accumulate at the centrifugal pole. The metachromatic particles, as seen with dye concentrations of 1 part toluidine blue in 100,000 parts sea water, are small (about 1/4 to 1/2 micron in diameter) and stain heavily. With higher dye concentrations, many particles appear as stained vesicles more than a micron across. This is especially true with neutral red.

Some of the unfertilized eggs are parthenogenetically stimulated by the sucrose "pycnotic" barrier. In such cases particles are closely associated with the asters formed (see Figure 2).

2) *General behavior of particles in eggs after germinal breakdown.* After germinal vesicle breakdown to at least second cleavage, the metachromatic particles can be found in two locations: a layer at the centripetal end of the clear zone, and a layer at the centrifugal end of the yolk area. These general locations can be seen in all four types of staining experiments performed (*i.e.*, with relation of staining to centrifugation times), and, indeed, the localizations appear identical, although, in general, the closer the observation time is to the staining time, the *lighter* does the stain appear. The particles in both widely separated locations appear to have the same size and staining characteristics as the particles in uncentrifuged eggs (remember, however, the difficulties of observation in the yolk area). With high dye concentrations, 1 part or more toluidine blue in 50,000 parts sea water, the particles in the centripetal layer may be considerably larger than those in the centrifugal layer.

In addition, when centrifuging eggs at the stages in which polar bodies are given off or after syngamy, one sees two other phenomena in most if not all eggs; one, there is a thin, clear zone separating the centripetal layer of particles from the lipid cap (this is not present when pronuclei have formed); two, one often sees very tight knots of particles in this centripetal zone of particles. If one follows these tight aggregates for 10 to 15 minutes, one gradually sees the mass grow larger, the radial arrangement of grape-like masses of stained particles form and, in general, the development of all the activity characteristic of asters. It would appear that the centripetal particles represent the β-particles of Pasteels (1955), by both their location in the centrifuged egg and their behavior relative to the asters. It is clear that these particles are directly stainable, since they were seen at all times in the mitotic cycle, at the most 4 minutes after the beginning of staining and, in one experiment, at 2½ minutes after the beginning of staining (see Discussion).

The results of staining centrifuged eggs at various times in the cleavage cycle differ somewhat from those with unstratified eggs so stained. If, at first cleavage, one compares an unstratified egg stained before fertilization with one stained at this stage, one finds in most batches of eggs an extensive "diffuse" stain in the latter, not found in eggs stained before fertilization, which may obscure the astral aggregation of metachromatic particles unless the eggs are compressed. In addition, the astrally aggregated particles appear to stain more lightly in eggs stained at first cleavage. The eggs stained in these two ways thus look different in gross appearance, one egg clear, the other "diffusely" stained. The centrifuged eggs look much more similar in this regard, although the eggs stained after centrifugation at first cleavage stain less heavily. In watching the latter, it can be seen that the centripetal particles (β-granules) increase in color intensity with time. This increase in intensity of β-granule staining, coupled with a destaining of the vitelline membrane and jelly coat, probably contributes to the differences in "diffuse" staining seen in the unstratified eggs.

It is clear that after germinal vesicle breakdown two groups of stained particles can be seen. We saw that this division of particles into two groups with the centrifuge occurred in eggs which had been stained before fertilization and then stratified at first cleavage. However, such eggs when observed before stratification appear to be almost completely devoid of particles anywhere in the egg except in the astral regions, and it, thus, is important to know where the two groups of stained particles come from. To get at this problem we centrifuged eggs lightly at 4380 g for 1 minute at the first polar body stage and at first cleavage. This partially stratifies the egg (a definite hyaline zone is not present, although lipid, yolk, and cortical granule layers are). In eggs stained before fertilization, the spindles with attendant stained particles were very clear. In many such eggs no metachromatic particles could be seen in the centrifugal yolk area, although this was not universally true. If eggs were stratified first and then stained, the particles in the asters were seen but were lighter in stain than in the previous case. In addition, particles were seen in the centrifugal zone, but it was impossible to determine if there were more or fewer than in the first experiment. In eggs from these same batches, centrifuged at the higher speeds and longer times discussed earlier, the two groups of particles, centripetal and centrifugal, were clearly seen.

j. *Other staining methods*

Our results with both the Nadi reaction and Janus Green B staining can be simply stated: at all stages in mitosis the stain appears only in the mitochondrial layer in centrifuged eggs, and in non-centrifuged eggs we find only a uniform distribution of stain in the cytoplasm external to the spindle (for a different result, see Dalcq *et al.,* 1956). This result agrees with that which would be predicted from our electron micrographs of eggs at various mitotic stages, since in these micrographs, no specific localization of mitochondria can be seen in uncentrifuged eggs at any stage of mitosis. In centrifuged eggs, the electron microscope shows mitochondria to be gathered primarily into a layer just centripetal to the yolk (both in fertilized and unfertilized eggs), although they can occasionally be seen in the lipid and yolk layers (see also Pasteels *et al.,* 1958).

Discussion

The events of early cleavage in *Spisula*, which we have set forth above, are similar in many details to those described in other lamellibranch eggs such as *Unio* (Lillie, 1901), *Mactra* (Kostanecki, 1904), and *Barnea* (Pasteels, 1930). A discussion of all aspects of the cytology of these stages (which can be found in Raven, 1958) would be out of place in the present paper and we shall concentrate only on some of the more puzzling problems which these observations raise.

A problem which fits the latter category is that of spindle translation and oscillation in first cleavage, first reported by Lillie (1901) in the fresh-water clam, *Unio*. Lillie found no morphological structure revealed by his technique which could account for the precision and movement which the first cleavage spindle possesses. He felt, however, that there must be some invisible (but by no means mystical) organization of the cytoplasm which imposes its influence upon the spindle, and that this is part of that more general organization which determines the mosaic character of the egg. That he observed the spindle oscillation as well as translation seems clear from his statement that "the position of the spindle is controlled through the cytoplasm as a needle in a magnetic field oscillates until equilibrium is attained" (Lillie, 1901: page 255).

Spindle movements in the gastropod, *Crepidula*, were described by Conklin (1902, 1912) and were ascribed by him to the influence of cytoplasmic currents. The peripheral migration of the first polar body spindle was thought to be due to an axially directed vegetal-to-animal current which moved the spindle to the animal pole. The characteristic alternation of spindle directions in third and later cleavages in *Crepidula* (and presumably in other *Spiralia*) was considered to be due to currents which themselves altered direction with cleavage in late anaphase. Experimental interference with those currents appeared to stop cleavage (Conklin, 1938).

A cortical influence on spindle displacements appears from the work of Pasteels (1930, 1931) on *Barnea candida*, and, indeed, Raven (1958) feels that the primary influence originates in the cortex. This influence presumably affects the spindle through the cytoplasm, possibly through an influence on cytoplasmic currents.

Our own observations on spindle movements, both visually (with the microscope) and in time-lapse movies, make it clear that cytoplasmic displacements and spindle movements are correlated events. However, the dissection of cause from effect in these phenomena is by no means obvious. Indeed, in many time-lapse movie sequences of spindle movement, the rapidity of its inception and apparent directedness of its trajectory leave a definite impression of an object pulled, rather than pushed, through the cytoplasm. It is not impossible that a morphological connection of a temporary nature may attach the spindle to the egg cortex, and that contraction of this connection is the event which displaces the spindle toward the cortex.

Some indirect evidence for such a view can be cited. First, the egg cytoplasm is capable of rapid localized contractions. These can be seen in *Spisula* eggs which become amoeboid after membrane removal with alkaline isotonic NaCl (similar amoeboid phenomena in eggs have been reported by Lillie, 1902, and Monroy, 1948, in annelids; by Pasteels, 1930, and Kostanecki, 1904, in molluscs; and by Harvey, 1938, and Moser, 1940, in echinoderms). Very rapid "twitches" may occur in some parts of the eggs while other parts appear quite fluid (*i.e.*, are flowing). Second, a close relation appears to exist between the cortical granules and the spindle ends. This is a normal phenomenon in later (*i.e.*, third and on) cleavages and will be reported in detail in a later publication. In addition, in eggs treated with alkaline isotonic NaCl, as early as first cleavage cortical granules (as distinguished by size, shape and staining properties) may appear tightly aggregated about a single pole of the spindle (which in these cases does not migrate) (for such a relation in *Mactra*, see Kostanecki, 1904), indicating connections of the spindle pole with the cortex which allows the granules to be pulled to the pole under the experimental conditions described above. Such connections (if they exist) need be no more permanent than astral rays (which they may indeed be). Finally, if such connections consisted of local gelated regions of cytoplasm (fibers, rods), their movements relative to the surrounding cytoplasm might, indeed, have been described as cytoplasmic currents. All in all, one need not be bound to the idea of a fluid current as being the motive force in spindle movement.

Existence of particles

In any study using the techniques here discussed a primary question is the existence of the particles prior to treatment. The controversies concerning the origin and existence of the "vacuome" (Hovasse, 1956) visualized after neutral red staining make any such staining techniques guilty, until proven innocent, of the production of the particles by the techniques themselves. This is the more so, since a type of reaction to injury in the egg is the formation of vacuoles (Heilbrunn, 1956).

We have briefly discussed the vital staining experiments of Pasteels, Mulnard, and Dalcq in the introduction. In addition to this work with the living cell, these authors used the Gomori acid phosphatase technique and the Alcian blue technique for acid mucopolysaccharides, and were able to show, in several invertebrates, that acid phosphatase and acid mucopolysaccharides in the egg showed the same localization changes as those undergone by the dye particles in the vitally stained egg (Pasteels and Mulnard, 1957; Pasteels, 1958; Mulnard, 1958). We have confirmed some of these observations with *Spisula* and will report them at a later date. These histochemical results indicate that, at least, there is a moiety (or "plasm") of the egg which predates any treatment with dye, and which follows the centrosomes, although it does not prove that this moiety is particulate. We will discuss this problem after first comparing our results with those of the Belgian school.

Comparison of the present work with that of Pasteels, Mulnard, and Dalcq

We have already indicated that the above authors feel that there exist in the egg two types of particles: a small, lightly staining metachromatic one, called the α-granule (or α-mitochondrion) which can be present and formed at any time in the cleavage cycle, and a larger, deeply staining one which appears in the cell only at certain specific periods in the "life" cycle of the egg (although it may exist before it can be stained). In the molluscs, *Barnea* and *Gryphea* (Pasteels and Mulnard, 1957), the sea urchins, *Psammechinus miliaris* (Pasteels, 1955) and *Paracentrotus lividus* (Pasteels, 1958), and the ascidian, *Ascidiella aspersa* (Dalcq *et al.,* 1956; Dalcq, 1957), this period is primarily that during which copulation of the pronuclei is beginning to occur, although some particles may be formed at the time of the second maturation division. In *Chaetopterus pergamentaceus* (Mulnard, 1958) the particles begin to appear at the first maturation division.

Several bits of evidence are adduced by the Belgian workers for the existence of two particles, the most telling being the so-called indirect staining of the β-granules. That is, the observation that eggs stained at cleavage show only a diffuse light granular stain (α-particles), the astral location only occurring at the next cleavage and then in particles larger and more darkly staining than the first. In *Chaetopterus,* however (Mulnard, 1958), there appears to be some question as to whether the β-particles are not directly stainable since in several cases Mulnard reports their appearance after staining "à un stade où les granules β existent déjà en grande nombre" (Mulnard, 1958, page 657), and, therefore, their direct stainability. He feels, however, that this is probably due to their rapid formation during the five-minute staining period he used and during which time material may be transferred to the β-granules from α-granules.

Two further bits of supporting evidence for the two-particle idea come from vital staining experiments and from histochemical tests. Thus, Pasteels (1958) claims that in *Paracentrotus* (the one exceptional species, so far) the α-granules do not stain with the acid phosphatase technique (although the β-granules do). In addition, the α-granules stain with neutral red but the β-granules do not, and thus neutral red particles do not show an astral location. This last result should, however, be contrasted to the neutral red results obtained by Iida (1942) in a Japanese species of sea urchin (species not given) and by Kojima (1959a) in three species of sea urchins.

It is clear from the above discussion that the same basic phenomena have been described by ourselves and the Belgian group. It is equally clear, however, that differences exist. In view of the variation in results which that group have themselves reported in different species, it can be assumed that at least a part of our differing observations can be traced to different materials.

Our own vital staining work supports the idea that the particles pre-exist the treatment with dye. Thus, in all cases staining of eggs immediately before or immediately after centrifugation at any point in the cleavage cycle gives essentially the same result (keeping in mind the precautions noted in the section on observations). Indeed, if the dye were causing the formation of particles (or vacuoles) from a pre-existing, but non-particulate, moiety of the egg, one would expect the vacuoles to have a different relative specific gravity from this "precursor" and, therefore, to show a different centrifugal behavior from it, which the experiments discussed in Observations, section II i, show to be false. This change in relative specific gravity is, however, what occurs in the eggs heavily stained with methylene blue, where the particles are deliberately caused to swell, and where they centrifuge to the upper stratum of the yolk layer.

A question of great interest is that of the number of types of stained particles present. Our centrifugation experiments described earlier indicate, in agreement with the work of the Belgian group, that after germinal vesicle breakdown, two groups of particles are present, one group centrifuging to the centripetal part of the hyaline layer (for more detail, see observations) and one, to the centrifugal end of the yolk layer. These particles appear to be about the same size (they are too small for very accurate measurement) and to stain with approximately the same intensity if examined 10 to 15 minutes after staining. The location of the particles in the stratified egg seems to agree with the locations of the α- and β-granules of the Belgian group. The fact that the centripetal particles migrate into the asters in redistributing eggs leaves little doubt of this identification. We may, therefore, identify our centripetal particles with the β-granules of the Belgian group, and the centrifugal particles with their α-granules. With this identification it is clear that a fundamental difference of observation exists between the work reported here and that of the Belgian school, namely, that we find the β-granules to be stainable directly and to be present and identifiable at all points in the cleavage cycle after germinal vesicle breakdown. This is supported by direct observation on compressed eggs stained with low dye concentrations at appropriate times in the cleavage cycle, although, as was mentioned in the section on observations, the difficulties due to "diffuse" staining must be carefully overcome. In addition, the observable fact that β-granules increase in depth of stain with time must be taken into account.

A further difference between our results and those of the Belgian group (although one fraught with some observational difficulties as previously discussed) derives from the results of centrifuging eggs at first cleavage, which were stained before fertilization (or soon after). Eggs at first cleavage show essentially no stained particles elsewhere in the egg other than in the asters. In lightly centrifuged eggs one finds many cases in which metachromatic particles are found only in the (centripetally located) asters. However, such eggs stratified with the higher forces show the two groups of particles, α- and β-granules. It would appear, therefore, that both α- and β-granules, in *Spisula*, show an astral aggregation, which is maintained under low, but disrupted under high centrifugal forces.

To reiterate, then, in *Spisula* eggs one finds two sets of particles, both directly stainable with toluidine blue (or neutral red, etc.) and both of which show the migration into the asters. What the relationship between these particles is (and to what degree the centrifugal behavior of the particles represents a real difference in their nature) we cannot say from our observations. Our work neither lends support nor in any way detracts from the idea of the Belgian groups that the α-granules are precursors to the β-granules. However, in *Spisula,* if this is so, the process of β-granule formation must be continuous throughout the cleavage cycle, with the β-granules so formed, directly stainable.

Direct confirmation of the existence of the particles in the untreated living cell would still be highly desirable. Dalcq *et al.* (1956) report that particles showing the behavior under discussion can be seen, with the phase microscope, in the living egg. However, using both phase and interference microscopy, with and without the immersion media earlier discussed, we have not been able to confirm this observation, although it is true that occasionally dark particles (in dark contrast phase), or particles of a slightly different hue from other particles in the cell (in interference microscopy), appear near the centrosomes in living, unstained eggs. Unfortunately, the presence of many highly refractile granules in eggs makes such observations unreliable, since even in compressed eggs halos make detailed observations difficult in phase microscopy. Reversed color effects from particles not in the image plane make interference microscope images also unreliable in this case. In addition, the contrast in a phase microscope is a function of the optical path difference between an object and its surround (Bennett *et al.*, 1951), and, since the average refractive index of the cytoplasm outside the asters and spindle is different from that of the asters and spindle themselves (Ross and Rebhun, unpublished experiments; Mitchison and Swann, 1953), it would be expected that particles near the asters would show some contrast difference with those elsewhere. These disappointing results may simply mean that the optical path difference between these particles and the cytoplasm and that between other cell particles and the cytoplasm is about the same and the particles are therefore not distinguishable by this property alone.

A similar disappointing result has so far been obtained by us with the electron microscope. Our work (Rebhun, 1958) and that of Pasteels *et al.* (1958) seem definitely to remove mitochondria from consideration as candidates for the stained particles, reversing a previously stated suggestion (Pasteels and Mulnard, 1957; Dalcq *et al.*, 1956).[3] Pasteels *et al.* (1958) feel that in centrifuged eggs of the sea urchin, *Paracentrotus lividus*, they can see "Golgi" bodies in the layer to which the acid phosphatase positive, metachromatic β-granules go. Unfortunately, they present no electron micrographs to support this claim. In addition, they do not see these bodies specifically associated with the asters. In our own electron micrographs of *Spisula* we can occasionally see at least two different types of particles possibly associated with the asters, one of which looks superficially like Golgi bodies in having closely packed concentric lamellae surrounding a vacuole. However, this may merely indicate a complex lipid cortex such as occurs in the methylene blue, brilliant cresyl blue and Nile blue staining phospholipid particles in pulmonate snail neurons (Chou, 1957; Ross and Chou, 1957). Indeed, in electron micrographs of such neurons, bodies, such as described above in electron micrographs of *Spisula* eggs, do occur (Ross, personal communication). The second type of particle resembles the multivesicular bodies in rat eggs (Sotelo and Porter, 1959). All in all, the electron microscope is disappointingly vague on the questions relevant here. We shall, however, report these results in detail at a later date.

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[3] I would like to clear up a misunderstanding concerning these results which apparently occurred during a conversation with Dr. Mulnard and which I discovered only upon reading his paper (1958). My position concerning the existence of the particles is there reported as one of scepticism which, it is claimed, stems from electron micrographs of heavily stained eggs (method I). These micrographs do, in fact, show vesicular bodies not seen in unstained eggs, with single or double membranes outside and vesicles or "cristae"-like (Palade, 1953) objects within. It was clear to me, however, as discussed in this paper, that these large bodies arise from the prolonged action of dye upon a pre-existing substratum. Among the possible particles which might be involved as substratum, I considered at that time, a particle with the morphology of mitochondria but with different enzymatic properties, but realized this to be very hypothetical. I now reject this possibility. I have never thought these particles to be degenerated, "ordinary" mitochondria as stated by Mulnard (1958).

Some observations which may have a bearing on the problem of the general existence of the particles other cells, are those of Bloom *et al.* (1955, Fig. 2) on newt heart fibroblasts. The movies from which this paper is taken show many dark particles (called "fat droplets" by the authors) which migrate to the centriole region of the cell in prophase and show a distribution in division very similar to that described above in eggs. Indeed, there appears to be enhanced movement of many of the particles and an aggregation into grape-like masses during division, similar to that described in eggs. Histochemical studies of these cells, however, have not been reported.

Finally, Holt (1957) showed that esterase and acid phosphatase in mitoses, after formalin fixation, in regenerating rat liver showed the same localization changes that the basic dyes and acid phosphatase do in eggs.

Preliminary identification of the particles

Work has already been quoted which indicates that these particles are not mitochondrial in nature. We have also indicated our attitude of constraint (not, however, negation) toward the suggestion that the β-particles are themselves Golgi bodies. The suggestion remains (see Mulnard, 1958) that the α-granules are lysosomes in the terminology of de Duve (1957), this being supported by the fact (ignoring Pasteels' (1958) negative result in *Paracentrotus* for α-granules, for the moment) that the α-granules contain acid phosphatase. The lysosome suggestion is strengthened by the observation of Holt (1957) that acid phosphatase in liver parenchymal cell mitosis shows the same behavior as our particles do in living eggs, since the lysosome concept originated from particles obtained from liver. Our preliminary work with egg homogenates indicates that the acid phosphatase of the egg is sedimentable at relatively low speeds, rather than soluble. However, we find no evidence that the activity is increased by treatment with Triton-X, aging at 37° C., freezing and thawing, or treatment with distilled water. Thus, the egg particles lack a fundamental property of lysosomes, *i.e.,* a *releasable* acid phosphatase. These results will be reported in detail at a later date.

Motion of the particles

As has been pointed out, the particles may move with great rapidity and in fixed directions. This motion is superimposed on a Brownian motion similar to that undergone by other particles in the cytoplasm. The characteristics of the motion of the stained particles are: (a) they may move with velocities up to several microns per second; (b) two particles within a few microns of each other may move rapidly in opposite directions; (c) the rapid movements are, generally, radial to the centrosome, either towards or away; (d) not all particles move rapidly at any given time, *i.e.,* some may be undergoing ordinary Brownian motion; and (e) these motions are *not* participated in by most other inclusions in the cell (the motions of cortical granules in later cleavages may be an exception). Any mechanism proposed to explain these phenomena must explain them all.

There are several types of forces which might be invoked to account for these events. For example, Bjerknes (see Schrader, 1953) published accounts of differential movement of particles relative to each other on fluids in which standing waves were set up by two pulsating spheres. The movement of the particles towards or away from the spheres depended on the phase of the vibrating spheres and the density of the particles relative to the fluid medium. This phenomenon has been used in models of anaphase separation of chromosomes (Schrader, 1953), and Pfeiffer (1956) has published accounts of supposedly pulsating centrosomes. We feel, however, that much more evidence for such standing waves or pulsating centrosomes would have to be gathered before such a mechanism could be considered as causing particle movement in eggs. In addition, the α- and β-granules appear to participate in the movement although their centrifugal behaviors, and therefore densities, are very different.

The intermittent growth of the astral fibers was postulated by Iida (1942) as causing the motion of the particles. This requires the particles to be attached to the astral rays in some way. Their normal Brownian motion, however, would seem to argue against such an attachment (although the fact that they centrifuge with the asters might support it). Also, the net direction of motion of the particles during mitosis is into the asters at a time when the asters are growing and, therefore, moving out. Finally, there is no evidence of a shortening of astral rays during their growth period as is required to pull the particles into the asters on the above model.

Cytoplasmic currents might be invoked as agents sweeping the particles into the asters. Conklin (1902) and Chambers (1917) considered the aster to be essentially composed of such streams. We feel, however, that certain observations speak against this notion, namely, the motion of some stained particles relative to nearby stationary ones, and, more particularly, their motion relative to yolk granules and mitochondria in the same region which remain stationary (apart from Brownian movement). Currents should be relatively indiscriminate in moving small particles of approximately the same size. Moreover, the motion of some of the particles is so rapid, and starts so suddenly, that it is difficult to imagine currents being involved. Finally, the same particle may be rapidly "jerked" into the aster and just as rapidly reverse its movement, although the net direction is inward as cleavage proceeds.

In connection with the last observation, we suggest the possibility of mechanical connections of some sort, possibly contractile fibers or gel streams, connecting the stained bodies to the centers. Contractile fibers of this nature have been postulated as being involved in the motion of echinochrome granules in *Arbacia* (Parpart, 1953). After fertilization, these particles move radially into the egg cortex (McClendon, 1910). In movies of this phenomenon, it is clear that the motion of echinochrome granules has exactly the same five properties as listed for the metachromatically stained particles, if we replace the word "centrosome" by the words "egg cortex" in (c) above (A. K. Parpart, personal communication).

We suggest, again, as for the case of spindle movement, that contracting gel streams, similar to those described by Allen (1955) in amoebae, might very well be involved in these motions, and that such streams might easily be described as cytoplasmic currents.

A detailed study of the relative movements of individual particles of different types at different times in the mitotic cycle, similar to the study of Hiramoto (1958) on cleaving *Clypeaster* eggs, would be most useful in deciding between some of the suggestions discussed above.

Function of the particles

Some recent work has thrown interesting light on some possible functions the particles may perform. Marsland (1958) has extensive evidence from pressure centrifugation work on premature furrowing in *Arbacia* eggs that a factor from the nucleus and one from the metachromatic particles are involved in the initiation of the cleavage furrow. This work is supported by the beautiful work of Kojima (1959a, 1959b) which indicates that halves of eggs obtained by splitting with the centrifuge, cleave or do not cleave depending upon whether they do or do not receive, during centrifugation, particles such as we have been discussing. In the sea urchins *Temnopleurus* and *Mespilia* stratified but not broken eggs contain particles in the centrifugal end and it is this end which cleaves subsequently. Other evidence was adduced by Kojima in support of the hypothesis that the particles are involved in cleavage initiation.

SUMMARY

1. Techniques for vitally staining oocytes of *Spisula solidissima* with certain basic dyes are described.

2. Such staining reveals small particles in the cytoplasm of the egg.

3. After fertilization the particles show very specific movements and localization changes which can be described by saying that the particles follow the centrioles through cleavage.

4. Spindle movements and position changes can easily be followed and are described in detail.

5. The results are discussed in the light of related work in the literature.

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WOUND HEALING PROCESSES IN AMPUTATED MOUSE DIGITS[1]

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For obvious reasons the healing of wounds has been the object of study by many biologists and medical researchers. The literature is enormous and basic information concerning the subject can be found in many excellent reviews by Marchand (1901), Arey (1936), and Cameron (1952), and has been incorporated in text books of Histology, Maximow and Bloom (1957), Surgery, Harkins (1957) and Pathology, Robbins (1957).

Difficulties inherent in mammalian material and the desire for uniform techniques in the study of wound healing have caused the non-clinical investigations to be concentrated upon a limited variety of wounds. Studies of epidermal and dermal healing have been generally confined to skin wounds made on the backs of rats, Lindquist (1946) or on rabbit ears, Clark and Clark (1953), and Levander (1950). The important problem of bone repair and regeneration has been studied predominantly in rib or leg fractures in rabbits, Ham and Harris (1956), cats, Blaisdell and Cowan (1926) or rats, McLean and Urist (1955).

But when one considers the problem of the type of wound that occurs after amputation of an appendage it is surprising to discover a lack of fundamental information of the subject. Systematic post-amputational wound healing has heretofore been studied only in the lower vertebrates, probably because of their capacity to regenerate limbs. Studies in man have been confined to gross observations and to attempts to hasten the process of healing via skin grafts and other devices (Slocumb and Pratt, 1944). Occasional studies have appeared in the literature that dealt with amputational healing in rat (Nicholas, 1926) or sheep fetuses (Barron, 1945); however, even these biologists confined themselves to gross observations. We have been unable to find any description concerned with the histological process of post-amputational wound healing in mammals.

This paper constitutes an attempt at a systematic investigation of the histological aspects of wound healing processes occurring in amputated mouse digits. Our objectives are two-fold: first, to describe the post-amputational events that lead to simultaneous and coordinated repair of skin, connective tissue and bone; secondly, to evaluate the findings from the point of view of the student of regeneration, and to ascertain whether there are any aspects of this process that might be comparable to those seen in regenerating vertebrates.

MATERIALS AND METHODS

The animals used in this study were four- to eight-week-old Swiss mice of a strain purchased from the Roscoe B. Jackson Laboratory in 1953 and maintained at this laboratory to the present time. They were fed a diet of Purina Lab Chow

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and kept at a temperature of 80° (± 2° F.) in plastic cages in groups which never exceeded seven mice per cage.

Previous attempts at a systematic histological study of post-amputational wound healing in mammals conducted at this laboratory (McIntosh, Amherst College Honor's Thesis, 1954) were confined to amputations made through the carpal, metacarpal or radius-ulna regions of the forelimb. It was found, particularly in the younger individuals, that the many bones at the amputation site were often severed at different stages of ossification, surely a complicating factor in an histological study. It is also reasonable to assume that a long bone amputated through the diaphyseal region might present a healing pattern different from the one amputated through the epiphysis. It was therefore decided to select for study the digit containing within each particular segment a single bone at a definite stage of development. The digits of the forelimb, because of their small size (roughly one millimeter in diameter), are ideally suited for an experimental and histological study.

The digits to be amputated were identified with the numbers I to V starting with the short digit which is homologous to the human thumb. The phalanges are numbered 1 to 3 from proximal to distal. Phalanx 3 is quite small and almost completely covered with a nail, so digital segments containing the longer phalanx 2 of the second, third and fourth digits of both forelimbs were selected for levels of amputation. Using the above numeration, a code system was devised to identify the amputation stump and host animal. Thus, for example, the third digit from the left forelimb of Case MCS 50, amputated through the second phalanx, was designated in our records and identified in this paper as Case MCS 50, L-2-III.

The mice were narcotized for the initial amputation with ether. Before fixation of the amputation stump, the animals were narcotized with a subcutaneous injection of veterinary Nembutal (Pentobarbital Sodium, Abbot) permitting a deeper narcosis. The doses varied from 1.8 mg. to 2.4 mg. depending upon the age, size and condition of the animal. The amputations were performed with small surgical scissors under the dissecting microscope. Due to considerable bleeding, no attempt was made to trim the bone stump to the level of the soft tissues; it is probably for this reason that in some cases a protruding bone complicated the healing processes. To avoid using an excessive number of animals, four to six digits were amputated on the same individual and these amputations were done either simultaneously or at varying intervals.

Before fixation, the digital hair was removed with a commercial depilatory (Nair). The stump of the digit was severed at the metacarpophalangeal joint and fixed in Bouin's solution for three days or longer and decalcified in Jenkin's solution for periods ranging from six to fourteen days. The embedding procedures entailed three paraffin changes and a 24-hour perfusion in the last paraffin bath at 56–58° C. All digits were sectioned at 10 micra. Difficulties in sectioning encountered were attributable to incomplete decalcification. Most sections were stained with Harris's modification of Delafields' hematoxylin and counterstained with orange G. Mallory's polychromatic stain was also used to show development of connective tissue fibers.

This investigation is based upon histological studies from 191 digits fixed at various times ranging from six hours to seven weeks after amputation.

EXPERIMENTAL RESULTS

Although, as mentioned in the introduction, the role of the epidermal, dermal and bony tissues in the healing of wounds has been well studied, it was desirable to re-investigate all these processes in the healing of amputational wounds. The healing stumps were fixed and studied histologically at six-hour intervals for the first two days, and daily thereafter for three weeks. The healing of epidermis, dermis and bone as observed in these amputational wounds will be discussed with particular emphasis upon the unique interrelationships between these tissues.

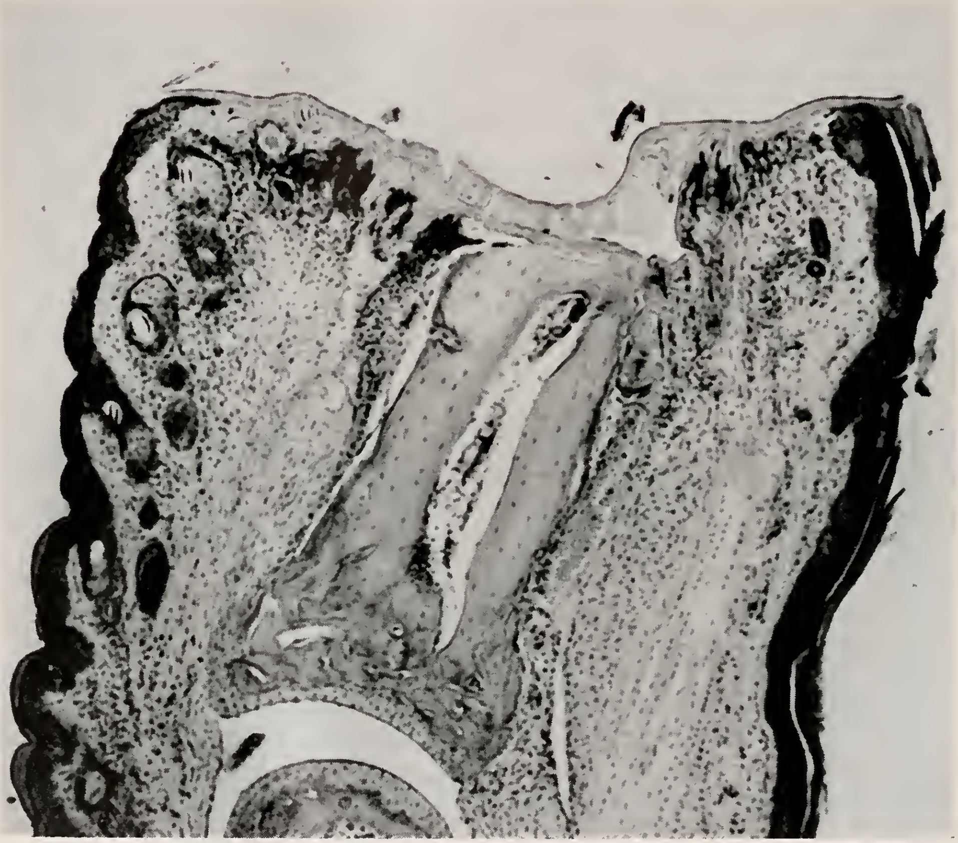

FIGURE 1. Photomicrograph of a sagittal section from a digit (Case MCS 52, L-2-III) fixed six hours after amputation. This case illustrates several aspects characteristic of the early wound healing process: (a) the entire surface of the cut digit is covered by a layer of blood coagulum with only a few blood cells in it; (b) beneath this coagulum can be seen large masses of white blood elements characteristic of this early inflammation phase; (c) the cut edges of skin show the beginning of epithelial migration and healing (90 ×).

For aid in orientation when viewing the figures, a sagittal section of a mouse digit illustrating its typical anatomy is presented in Figure 1 (Case MCS 52, L-2-III). The skin on the right side of the photomicrograph represents the volar surface, the left side representing the dorsal surface. The palmar or volar surface of the mouse digit is characterized by the absence of hair follicles and by a thick epithelium and dermis, while the dorsal epithelium possesses many hair follicles and is thinner than the volar. When a digit is sectioned horizontally the follicle distribution permits one to determine the exact position of the section. Amputation in this case was made through the mid-diaphyseal region of phalanx 2, and it is possible to observe the retracted extensor and flexor tendons on both sides of the bone.

The early hours of wound healing after amputation are characterized, according to Arey (*op. cit.*) and Robbins (*op. cit.*) by provisional closure with a blood clot and subsequent inflammation. Six hours after amputation (Fig. 1) the wound area is covered by a solidified blood clot beneath which polymorphonuclear leukocytes are agglomerating. By one day (Fig. 2, Case MCS 129, L-2-III), the extent of necrosis as indicated by the area of leukocytic activity has spread deeper into the tissues, particularly around the cut end of the bone. The polymorphonuclear leukocytes that characterize the early stages of inflammation begin to die off and they become extruded with part of the scab (Fig. 2). Subsequently monocytes and lymphocytes from the blood and young macrophages from the tissues identifiable in large numbers for at least three days replace the polycytes.

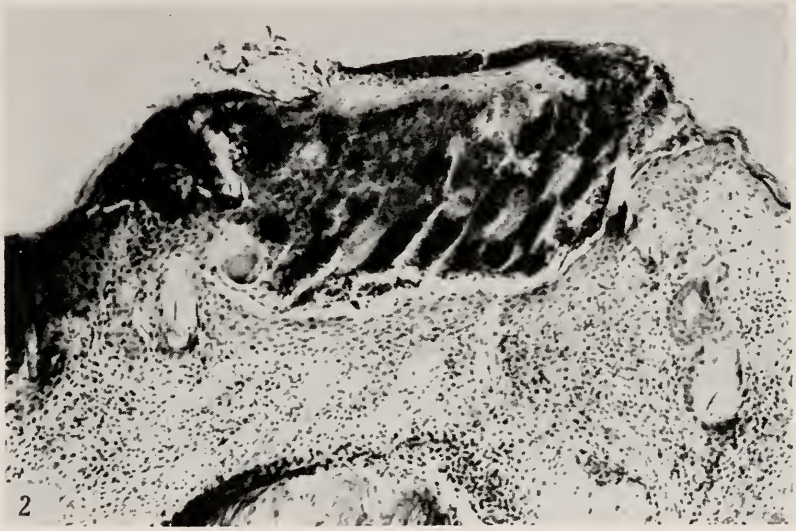

FIGURE 2. Photomicrograph of longitudinal section from a digit (Case MCS 129, L-2-III), fixed one day after amputation. A continuous layer of epithelial cells covers the amputation surface and it separates from the stump the large scab composed of clotted blood and various tissue debris. Inflammation at this stage has spread into the stump, and large masses of leukocytes have accumulated around the cut end of the bone (150 ×).

The first stages of epithelial migration and healing are in most aspects similar to those first described by Loeb (1898) in his classic work on epithelial wound healing. As seen in a digit fixed six hours after amputation (Fig. 3, same case as Fig. 1), the first cells to migrate derive generally from the granular layer of the epithelium. These cells rapidly swell, elongate and begin to migrate into and over the newly formed clot. The "syncytial protoplasmic layer" which according to Loeb (*op. cit.*) covers the clot or scab cannot be confirmed from the evidence of Figure 3, and from all the other sections we have studied. Rather, we have repeatedly observed an exudate (clearly shown in Fig. 3) of the type to which Weiss in several of his papers attracts attention (see last report on the subject, Weiss, 1959). We have made no histochemical study of the constitution of this exudate, but the photomicrograph clearly shows its orientational patterns (parallel to the amputation surface) so important for "contact guidance" (Weiss, *op. cit.*) of epithelial cells migrating over a semisolid substrate.

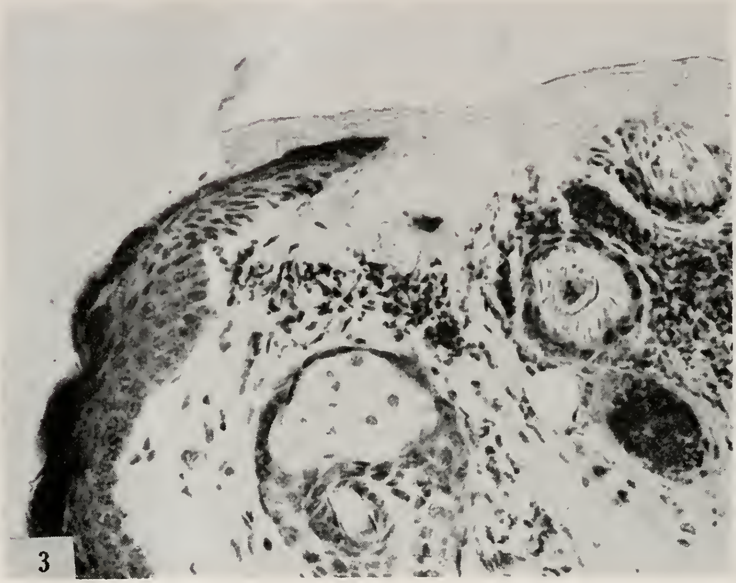

FIGURE 3. Photomicrograph of the upper left amputation site of Case MCS 52, L-2-III (shown in Fig. 1). The early migration six hours after amputation of the epithelium from the dorsal skin edge is shown. The migratory elements derive from the granular and lower horny layers of the epithelium, and the migrating wedge of cells can be seen invading the acellular exudate exhibiting fibrous structure. Beneath the clot large numbers of inflammatory leukocytes surround the hair follicles (300 ×).

The migration from the basal Malpighian layer proper takes place less rapidly. At six hours these cells are just beginning to swell and to elongate, and it is not until eighteen hours after amputation that a migrating wedge of epithelium from the Malpighian layer can be observed to infiltrate the area beneath the scab and the tissue debris. Once started this migration continues more rapidly, since one day after amputation a continuous layer of epithelial cells from the Malpighian layer can be found covering the area of tissue debris underneath the scab (Fig. 2). It is, however, only exceptionally that the epithelial covering is completed within one day. As a rule epithelial healing is observed in the majority of the digits only three days after amputation (eight out of the thirteen cases studied showed at three days a complete epithelial covering of the stump), in some other digits, undoubtedly due to protruding bone debris, this process is still further delayed. Complete epithelial repair is shown in Figure 4 (Case MCS 55, R-2-II) where the new epidermis shows early stratification. Also shown on the figure is a continued proliferation and migration of the Malpighian layer resulting in this and in other cases in a thick (12 cell layers) epithelial wedge over the cut end of the bone (see also Fig. 5, Case MCS, R-2-III). This local or general thickening of epidermal layers after amputation is a feature which closely resembles that described by Rose (1948) as a regular feature of epidermal growth in early regeneration in the newt.

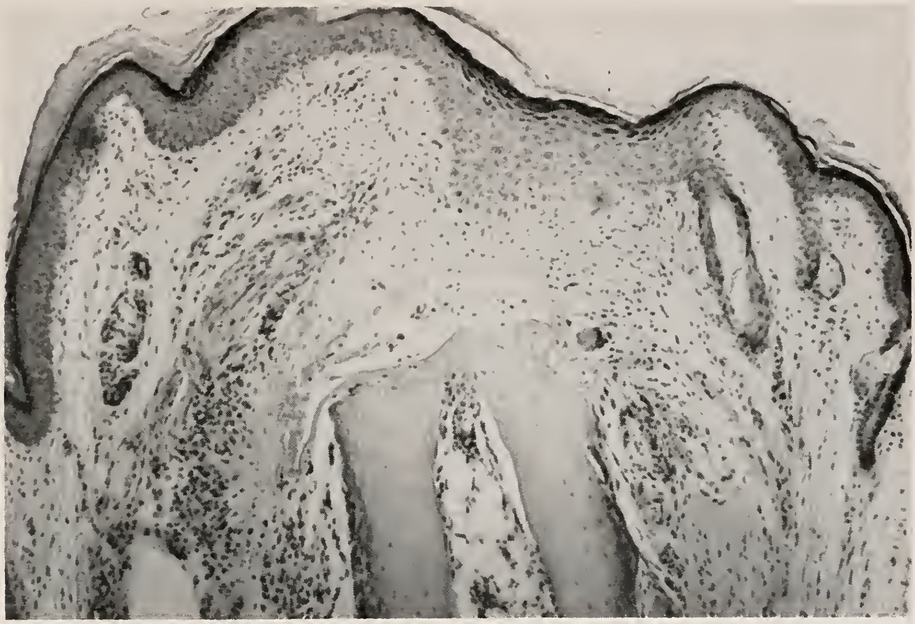

FIGURE 4. Photomicrograph of a longitudinal section from a digit (Case MCS 55, R-2-III), fixed three days after amputation and showing complete epidermal healing. Note: (a) the differentiation of epidermis into several distinct layers and the epidermal wedge with loosened cellular elements extending toward the bone; (b) between the covering epithelium and the bone are amorphous masses of clotted blood and tissue debris, while to the left of the bone masses of necrotic tissue can be seen; (c) individual fibroblasts identifiable by their spindle shaped form can be seen migrating anteriorly on both sides of the bone shaft; (d) the circular orientation of the tissues and the small area of epithelial proliferation, ahead of the phalanx reflects the contraction of the amputational wound (110 ×).

The reconstruction of dermal tissues begins three days after amputation. Fibroblasts from surrounding connective tissue and particularly from the regions of cut tendons can be seen streaming into the wound area. They proceed to intermingle with the pool of red cells and tissue debris that is characteristically found between the cut end of the bone and the new pad of epithelium. Once started, the proliferation and migration of fibroblasts proceeds rapidly and by six days (Fig. 5) a completed cap of fibroblasts separates the epithelium from the cut end of the bone. Seven days after amputation 13 of the 15 cases studied showed a well defined dermal pad.

Our observations of dermal healing concur with the previous descriptions of Arey (*op. cit.*) and Robbins (*op. cit.*): new blood vessels with at first indistinct walls accompanying the migrating fibroblasts become evident. Also, the first new collagenous fibers are seen at seven days in preparations with Mallory stain; consequently, the sub-dermal tissues become increasingly dense and fibrous until about two weeks after amputation when a characteristic dermal pad is found surrounding the end of the bone and the newly formed callus.

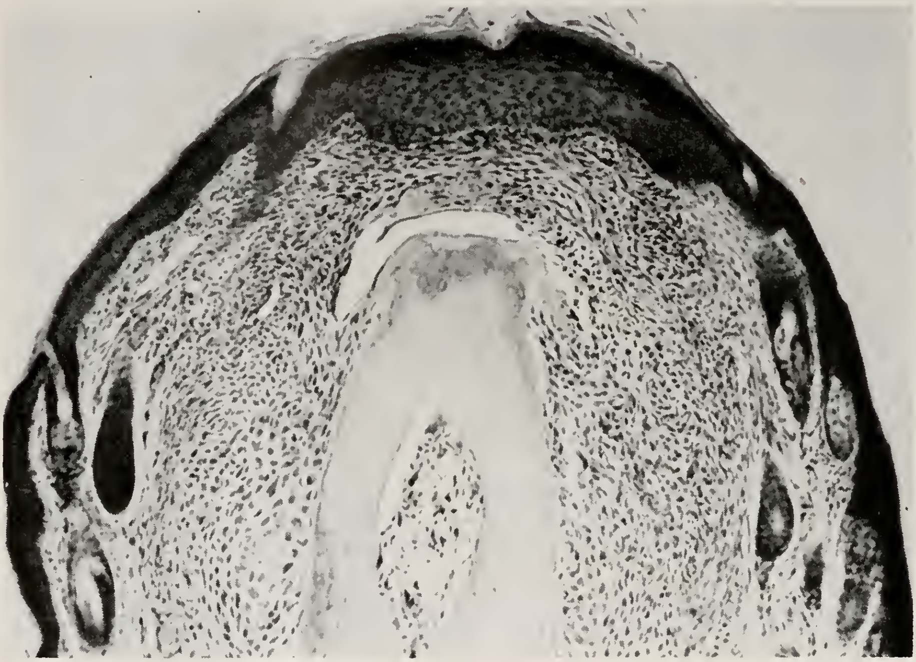

FIGURE 5. The photomicrograph of a longitudinal section from a digit (Case MCS 1, R-2-III) fixed six days after amputation shows: (a) the thick epithelial covering; (b) the connective tissue fibroblastic invasion into the amputation area resulting in a sizeable cap consisting of numerous layers of connective tissues; (c) the bursa-like cavity between this dermis and the end of the bone; (d) the extensive proliferation of periosteal elements with a large mass of cartilage cells on both sides of the bone (125 ×).

Reconstruction of bone tissue is also seen for the first time at three days, and our observations of the early stages of repair agree with the descriptions of McLean and Urist (*op. cit.*). The trauma of the operation and particularly the disturbance of vascular supply results in the death of osteocytes for varying distances from the distal end of the cut bone toward its proximal region. Empty bone lacunae such as shown in Figures 4 and 5 are represented under higher magnification in Figure 6 (Case MCS 100, R-2-III), fixed three days after amputation. The empty lacunae occupy most of the bone shaft; however, near the periosteum surviving osteocytes are discernible. The existence of surviving osteocytes at the proximal end of the bone shaft indicates that necrosis affects only the osteocytes located within the distal area of the bone affected by amputation. We have observed that the dead bone matrix is subsequently destroyed and removed by macrophages and giant cells, while the remaining bone tissue undergoes osteoclastic reorganization, which is particularly active around six days after amputation.

Reconstruction of new bone and the formation of a callus begins about three days after amputation as a proliferation of the osteoblasts forming the endosteal and periosteal coverings of the bone (Fig. 6). Once started, the proliferation of these osteogenic cells proceeds quite rapidly and by six days (Fig. 5) the early stages of callus differentiation become noticeable. On other sections not here illustrated, new bone trabeculae may be seen next to the old bone tissue, while the elements farthest away from the bone retain a fibroblast-like appearance. The cells which remain in an intermediate position generally go through a cartilage cell stage which is well illustrated in Figure 5 (to the left side of the shaft).

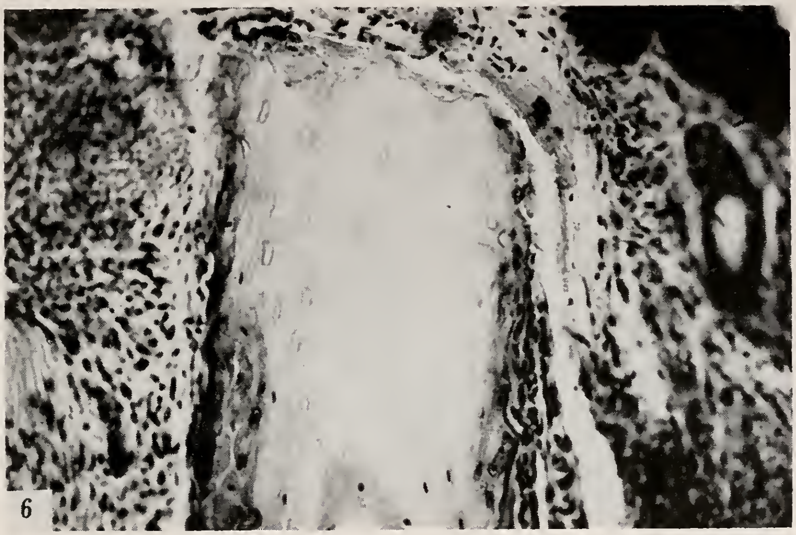

FIGURE 6. Photomicrograph of phalanx 2 from a digit (Case MCS 100, R-2-III) fixed three days after amputation showing: (a) the dead tip of the bone recognizable by the empty bone lacunae, while more proximally and toward the periosteal edges live osteocytes are visible; (b) the proliferation of the osteogenic cells in the periosteum on both sides of the bone (240 ×).

The size and shape of the callus that is formed varies according to the level of amputation. Observations indicate that amputation through the diaphysis of the bone results in a large callus with a characteristic intermediate cartilagenous stage, while if the epiphyses are transected a much smaller callus results. At the former level new bone trabeculae originate first near the bone and then at the outer edge of the callus. The cartilage cells intermediate between these two levels either disintegrate to make room to a highly vascularized zone that closely resembles the former hematopoietic marrow cavity, or they become calcified to leave thin bone trabeculae connecting the new outer shell of the stump to the old bone.

The formation of a new callus collar around the diaphyseal shaft is best shown in a cross-section of a thirteen-day-old amputation stage (Fig. 7, Case MCS 1, L-2-III). The outermost layers of osteogenic cells appear to be the largest and most active. Between the outer layer of new bone trabeculae and the old bone shaft can be seen perpendicularly arranged trabeculae, cartilage cells, and blood elements. Not indicated in this figure, but nevertheless observable in many sections, is a final callus that is much larger and of a more amorphous appearance than that typically described for healing in fractures (Ham and Harris, *op. cit.*).

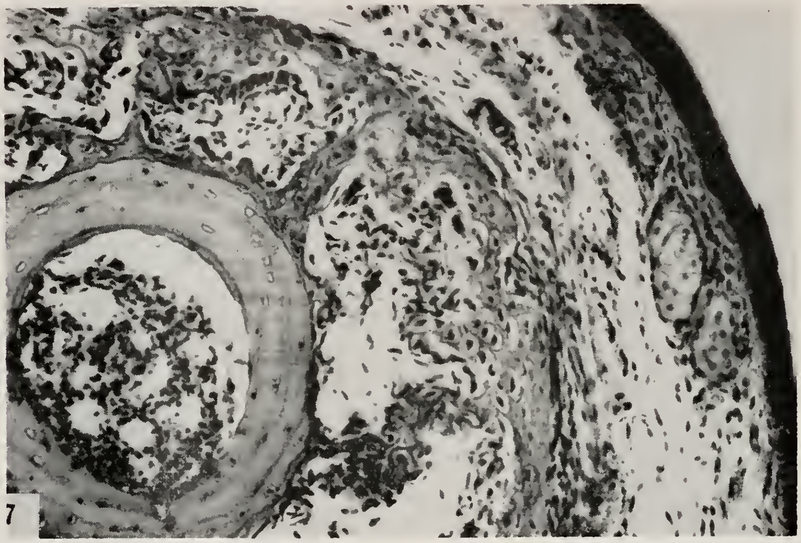

FIGURE 7. Photomicrograph of a cross-section from a digit (Case MCS 1, L-2-III) fixed thirteen days after amputation, showing the shape and extent of callus formation that follows amputation through the diaphysis. Observe the ring of new tissue formed around the old bone ring. External to the new layer of bone is a layer of large periosteal osteogenic cells. Between the peripheral region of the callus and the old bone new bone trabeculae, cartilage and blood elements are discernible (200 ×).

Amputation through and near the spongy epiphysis of the bone results in a different type of callus formation. Heavy proliferation of osteogenic elements is rare and, when found, these collect at the cut surface of the bone nearest to the diaphysis. Few cartilage cells are seen and remodeling appears to take place by osteoclasts and destruction of bone rather than by the formation of new bone trabeculae (Fig. 8, Case MCS 31, L-2-III).

Of particular interest in this and in other similar cases is the relative lack of reconstruction within the endosteal surface of the bone (Figs. 7 and 8), its main functions seeming to be confined to hemopoietic activities. Another interesting feature is the observation that the marrow cavity is invaded by connective tissue elements soon after amputation, and in these cases the processes of hematopoiesis revert to normal only after bone repair is completed.

The formation of bursa-like cavities. The aspects of post-amputational wound healing described so far concerned first the tissues immediately affected by amputation such as skin, and secondly the effects upon regions of the severed bone not directly injured by amputation. These are general reactions, encountered in any type of wound.

However, in amputational wounds of an appendicular organ such as a digit, features of healing are revealed which are peculiar and unique. Among these is the appearance of a pocket or tissue space which forms regularly over the end of the bone. At three days one observes a characteristic fluid area of necrotic debris and of free blood elements occupying the space between the cut bone and the covering epithelium (Fig. 4). With the onset of dermal healing, fibroblasts invade this area and five or six days after amputation the pool or pocket acquires a definite shape. The cavity or fluid space, similar to that seen in Figure 5 and which in many instances possesses a discrete synovial-like lining, closely resembles in histological features the bursae described by Black (1934).

Mention has been made in the literature of such an occurrence in amputated mammalian limbs: Nicholas (*op. cit.*) relates the formation of a bursa over the cut bone in a rat limb amputated in utero and fixed ninety days after birth, but no histological description was given. Nunnemacher (1939), studying the effects of partial amputation of epiphyseal cartilages in long bones of the same animal, describes and illustrates the appearance of a bursa within the connective tissues over the end of the cut bone. These observations are complemented by those of Urist, Mazet and McLean (1954) who describe the formation of a pseudo-arthroidal joint between the end of fractured bones that failed to appose. It does seem that a new bursa with a synovial-like lining is formed in amputational stumps in general, and that it is due to friction and irritation in much the same way as a pseudo-arthroidal joint is formed between the separated ends of a non-healing fracture.

Our observations differ from the above in the fact that the formation of a bursa-like cavity in amputated mouse digits was the rule rather than the exception: the fluid-filled area invariably appeared upon closure of the epidermis three days after amputation, acquired anatomical definition as a cavity in the midst of the invading fibroblasts, and then disappeared after the first post-amputational week. This last observation is in contrast to those made by the aforementioned authors in that bursal cavities here reported were transitory, while theirs were permanent.

Terminal aspects of wound healing in a mouse digit and in a frog limb

After the events described, the further post-amputational healing processes center around additional growth and thickening of the sub-dermal tissues. This latter thickening is effected by further fibrogenesis within the connective tissue, and the extreme degree of development of a pad of connective tissue in a digit fixed thirteen days after amputation is represented on Figure 8. The photomicrograph indicates that the digit was sectioned in a frontal, not a dorso-ventral plane, as the hair follicles are evenly distributed on both sides of the section. The figure shows that healing of the skin has been completed, the epidermal layers having reverted to their normal thickness; also, the capping of the end of the bone by the thick multi-layered pad of connective tissue suggests the end of growth of the cut phalanx. Observation of the dome-shaped connective tissue pad under higher magnification reveals its extensive vascular supply and also the "adult" appearance of the blood vessels. As was explained above, bone callus formation is only slight in this case because amputation was effected near the proximal epiphyseal region; however, periosteal cells are in the process of chondrofication and some new bone trabeculae have appeared. For all intents and purposes healing has been completed and little proliferative activity remains. It is clear from the examination of this and of similar slides from comparable post-amputational stages that no growth nor "regeneration" has occurred and that within a fortnight an amputated digital stump has reached true tissue equilibrium.

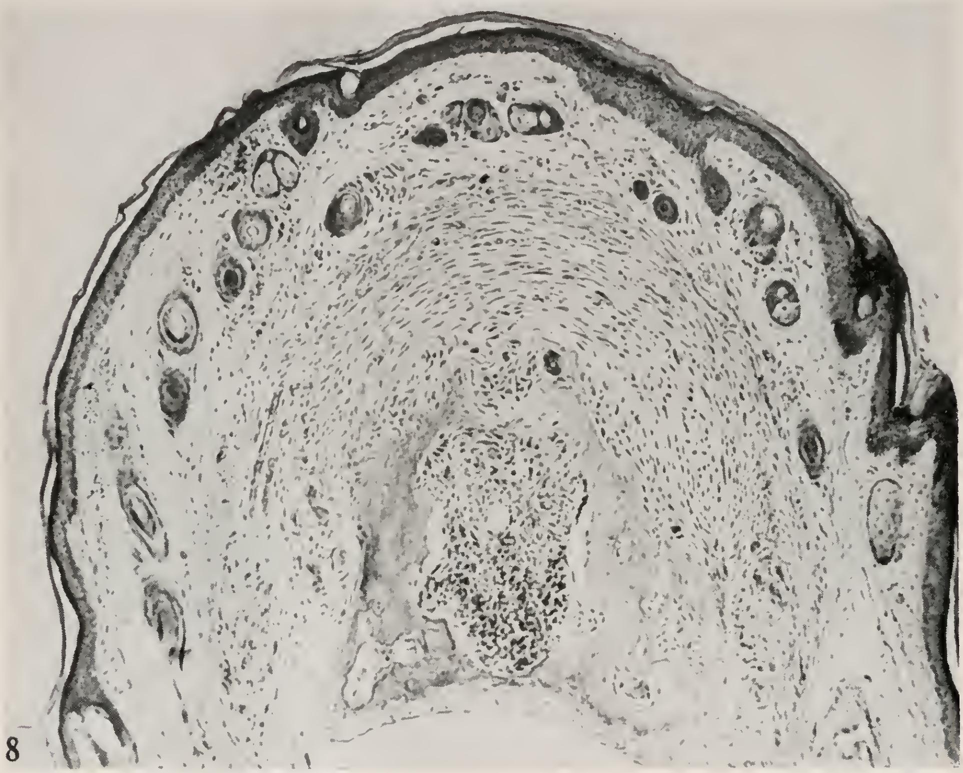

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FIGURE 8. Photomicrograph of a longitudinal section from a digit (Case MCS 31, L-2-II) fixed 13 days after amputation, illustrating completion of epidermal and dermal healing. The epithelium has thinned out and has resumed a normal appearance together with the completely reconstructed dermis and sub-dermal layer containing numerous hair follicles. In addition a thick cap of concentric layers of fibroblasts has developed over the cut bone and it separates the injured tissues from the fully re-formed skin. Within the connective tissue cap numerous new blood vessels are distinguishable. Since amputation was near the epiphysis there is little callus formation (110 ×).

This "final" stage of wound healing observed in an amputated mammalian digit may be compared to advantage with a similarly "terminal" stage of amputational wound healing in another, also non-regenerating vertebrate, a frog. A section from a forelimb of a post-metamorphic frog (*Rana clamitans,* 4.5 cm. long from snout to crotch), amputated through the lower arm and fixed 97 days after amputation will serve this purpose (Fig. 9). The longitudinal section from the lower forelimb shows: a completely regenerated skin as found on old amputational wounds; a cushion of sub-dermal formations including a fibroblastic pad tightly drawn over the terminal shaft of the ulna; finally, there is a well developed callus surrounding the severed shaft of the ulna.

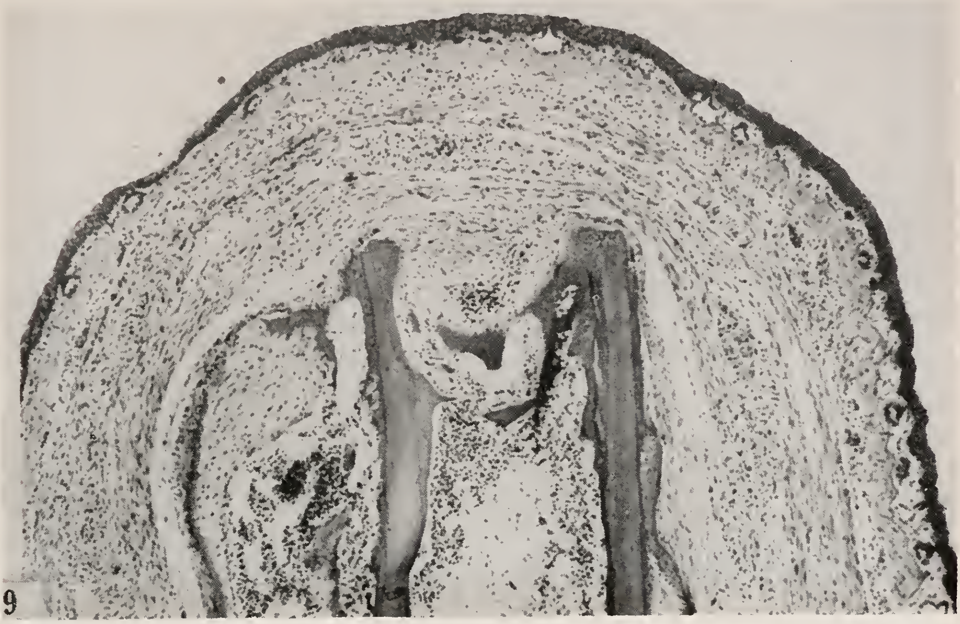

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FIGURE 9. Photomicrograph of a longitudinal section from a left forelimb of a post-metamorphic *Rana clamitans* amputated through the lower arm and fixed 97 days after amputation. The skin at the amputation surface is fully reconstructed as shown by the numerous functional skin glands, a thick basal membrane; the quiescent status of the epidermis is evidenced by the normal number of cell layers. The amputated distal end of the ulna is capped by a callus, most prominent on the left side of the bone. The callus formations have become a fully integrated part of the ulna; the transformation of a large portion of the proximal portion of it into bone trabeculae is clearly visible on the left; moreover, parts of the still cartilaginous callus have acquired a periosteal bone collar and a functional periosteum as well. Distally from the callus and the bone shafts concentric layers of fibrous connective tissues intermingled with muscle fibers offer the characteristic aspect of a non-regenerating limb (60 ×).

Because of the appearance of bony differentiations at the proximal end of the callus where it fuses with the periosteum of the bony shaft of the ulna it is inferred that the growth phase of the appositional callus is terminated. This observation is also supported by the numerous sites of insertion of muscular bundles and of tendons visible on the newly formed periosteum. All the conditions of a terminated organogenesis and of a nearly completed histogenesis are fulfilled. That is to say, the frog limb after the severe amputational trauma inflicted a hundred days prior to fixation has reacquired the tissue equilibrium of a fully developed organ at rest.

RECAPITULATION

The foregoing observations have shown that the healing processes following amputation in a mouse digit are unique, and that they differ from what has previously been described in non-amputational wound healing processes. This statement is based upon the following considerations.

After Marchand (*op. cit.*) it has been customary to classify skin wounds in two categories: firstly, those that show a small wound with little tissue loss and in which the epidermis heals over the wound without being preceded by granulation tissue formation are said to heal by primary intention; secondly, those that involve gross defects and extreme tissue loss involving particularly the dermis, heal more slowly, are characterized by accumulation of granulation tissue before epidermal healing may commence, and these are said to heal by secondary intention.

The wound produced by amputation of a digit certainly involves extensive tissue loss in an organ with a static morphological organization, and thus one would expect the features characteristic of a secondary type of wound healing. But all our observations concur to show precocious epidermal healing: as early as three days after amputation a completely joined epithelium was shown to exist; moreover, this epithelium was in many instances separated from the cut end of the bone by only a fluid area of tissue debris, and a completely re-formed epidermis was visible before any signs of dermal proliferation and repair had occurred. These features are certainly characteristic of primary wound healing, not of healing by secondary intention.

Lindquist (*op. cit.*), in an extensive study of skin wounds on the backs of rats, concluded that it was the contraction of the tissues around and beneath the wound which was more important for closure than was epidermal migration. Although the nature of the skin and of the soft tissues covering a mouse digit is different from that found on the back of a rat, our observations support the view of Lindquist that contraction plays an important role in the healing of these amputational wounds: while during the first day the amputation surface remains large (Fig. 1), by the third day of healing the wound surface is greatly reduced in size, and the actual extent of epithelial migration is small (Fig. 4). It also appears likely that the appearance of an amorphous fluid space between the healed epidermis and the cut bone is at least in part due to rapid epidermal closure. The subsequent organization of this space into a bursa-like cavity with its own lining is probably a secondary reaction to irritation within the amputation stump.

Another feature which distinguishes our observations from previously reported investigations concerns the bone callus. The illustrations shown offer evidence that in amputational wound healing the callus differs from that found in normally healing fractures. Following amputation through the diaphysis the formation of new bone trabeculae is irregular and diffuse thus producing a somewhat shapeless callus; however, amputation through the epiphysis results in mere destruction of the bone with little new callus formation. The irregular nature of the callus is no doubt due to the absence of a bone fragment in apposition to the injured stump, undoubtedly a condition which, in fractures, aids in the induction and organization of the proliferating elements into a more regular callus.

CONCLUSION

The investigator of wound healing processes (surely an unsatisfactory term) cannot help but be impressed and awed with the organism's ability to respond in such a complex fashion to the stimulus of a simple amputation. The loss and destruction of tissues determined by infliction of a wound stimulates in some as yet unknown way (see the thoughtful discussion on the subject of "New Tissue Formation in an Adult Mammal" by Abercrombie, 1957) first of all the migration and proliferation of the various cells within the amputation stump; but, more particularly, it also reawakens processes characteristic of ontogenetically earlier stages that lead to organization of these new and old cells into morphogenetically distinct tissues and organs. Note, for example, the formation of the large bone callus. the heavy cap of connective tissues of the end of the bone, and the appearance of a walled-in bursa-like cavity, transitory as it may be. Every one of these reparative and morphogenic mechanisms is surely under some form of systemic control, be it "wound hormones," nerves or endocrines (Abercrombie, *op. cit.*).

While it is surely essential for the student of regenerative processes to understand the causative factors involved in this post-amputational proliferation and migration of cells, the comprehension of the reasons for their precocious and apparently "final" differentiation into equilibrated structures appears still more imperative. To one accustomed to observing the properties of regeneration in urodeles where organological equilibrium is achieved a long time after amputation, it is most important to understand why amputation of a mammalian appendage leads within such a limited time to that tissue and organ equilibrium which is the very essence of arrest of growth and development.

For these reasons we have thought it rewarding to compare post-amputational wound healing in a mammal, with the type of healing seen in the amputated limb of a post-metamorphic frog, an animal that before metamorphosis possessed the ability to regenerate amputated limbs. The comparison of the histological features of amputated limbs from two non-regenerating animals offers convincing evidence, we believe, of fundamental similarities in their patterns of wound healing. In fact, there is nothing in the histological appearance of either of these two appendages that would suggest that one of these non-regenerating limbs would be more susceptible to respond to treatments that might awaken regeneration than the other. Yet, workers of the past two decades have shown that limbs of post-metamorphic frogs can be induced to regenerate: by surgical trauma (Polejaiev, 1936), chemical trauma (Rose, 1944), by augmentation of nerve supply (Singer, 1954) and finally by altering the systemic hormonal balance (Schotté and Wilber, 1958).

In view of the success of these experiments with frogs, animals with wound healing patterns that are strikingly similar to those of mammals, it is legitimate to expect that modifications of at least some aspects of wound healing processes may also be observed in mice under the influence of some experimental devices. Some such responses in healing patterns of mouse digits have already been obtained and they will be reported in a forthcoming paper.

SUMMARY

1. A systematic study of the early stages of amputational wound healing in mouse digits is presented. Digits were regularly amputated through the middle

phalanx and a total of 191 cases were fixed for histological study at periods ranging from six hours to three weeks after amputation.

2. The first phases of post-amputational healing, characterized by provisional closure of the wound by a blood clot and by subsequent inflammation, were found to be similar to non-amputational wounds, but differences were observed in the patterns of epidermal, dermal and bone healing.

3. Epidermal healing began six hours after amputation and was completed in most cases by three days. Dermal and sub-dermal connective tissues showed the first signs of healing not earlier than three days after amputation and it was generally completed one week after amputation. The observation that epidermal closure of the wound preceded any signs of dermal repair indicates that these amputational wounds heal by "primary intention." It was also observed that tissue contraction contributed to this type of healing.

4. Depending upon the level through which the phalanx was amputated, two types of callus formations were observed: (a) amputation through the diaphysis resulted in the formation of a large callus that was more diffuse and amorphous than those previously described for healing fractures; (b) amputation through the epiphysis resulted in very little callus formation, often concomitant with destruction of bone.

5. An unusual aspect of amputational wound healing in mouse digits was the appearance of bursa-like formations between the cut bone and the healed epithelium; at three days a fluid space formed which subsequently developed into a structurally distinct cavity with the morphological characteristics of a bursa. However, these structures were only transitory and they disappeared during the second post-amputational week.

6. Two weeks after amputation the mouse digit was found to be almost completely healed. Additional growth in the form of a heavy cap of connective tissues arranged in parallel layers enclosing the distal and lateral parts of the cut bone was an invariable feature of this healing. The rapid return to equilibrium of the tissues within the amputation site was compared to a similar type of healing observed in amputated limbs of post-metamorphic frogs.

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THE MORPHOLOGY AND LIFE-HISTORY OF THE DIGENETIC TREMATODE, ASYMPHYLODORA AMNICOLAE N. SP.; THE POSSIBLE SIGNIFICANCE OF PROGENESIS FOR THE PHYLOGENY OF THE DIGENEA

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CONSTITUTION OF THE GENUS ASYMPHYLODORA

The genus *Asymphylodora* was erected by Looss (1899) to contain *Distoma perlatum* von Nordmann, 1832, a parasite from the intestine of *Cyprinus tinca,* as type species. Rudolphi (1809) had suggested and Looss agreed that the species is identical with *Fasciola tincae* Modeer, 1790, from the same host. In the genus *Asymphylodora,* Looss included *Distoma exspinosum* Hausmann, 1896 from the intestine of *Barbus fluviatilus* and *Distoma imitans* Mühling, 1898 from the intestine of *Abramis brama. Distoma ferruginosum* von Linstow, 1877 from the intestine of *Barbus fluviatilus,* was considered identical with *D. perlatum. Fasciola globiporum* Rudolphi, 1802 was listed as *Distoma globiporum* by Rudolphi (1809) who stated that it included *F. tincae* and several other inadequately described trematodes. Looss (1894) had given a detailed description of *D. perlatum* and although he admitted the identity of this species and *F. tincae,* he did not make the correct taxonomic revision when he erected the genus *Asymphylodora.* Lühe (1909) made the combination *Asymphylodora tincae* (Modeer, 1790); he recognized the validity of *Distoma ferruginosum,* which he included with the three species selected by Looss as members of the genus. *Distoma punctatum* Zeder, 1800 from the intestine of *Cyprinus barbus,* was listed as probably identical with *Asymphylodora ferruginosum.* All of these worms were from the intestine of cyprinid fishes of Europe.

Subsequently, members of the genus have been reported from various parts of the world. Isaichikov (1923) described specimens from cyprinid fishes of the Kuban River as *Asymphylodora tincae kubanicum,* and this form was re-described by Markewitsch (1951). Ozaki (1925) described *Asymphylodora macrostoma* from several species of fishes in Japan. Ivanitskii (1928) described *Asymphylodora dneproviana* from fishes in the Ukraine. Witenberg and Eckmann (1934) found worms in *Cyprinus carpio* in Syria which they identified as *A. tincae.* After noting the variability manifested by different individuals, they declared that all previously described species were identical and accordingly, all other specific names were reduced to synonymy with *A. tincae.* Markowski (1935) described *Asymphylodora demeli* from *Gobius minutus,* taken in the coastal waters of Poland. He accepted *A. macrostoma* as a valid species and asserted that the action of Witenberg and Eckmann was not justified, since it was based on evaluation of published descriptions rather than on comparison of specimens. Srivastava (1936) described *Asymphylodora indica* from the intestine of the fresh-water fish, *Ophiocephalus punctatus* in India. Yamaguti (1936) described *Asymphylodora diplorchis* from *Pseudogobio esocinus;* a species which according to Yamaguti (1958) had been described by Takeuti (1936) as *Steganoderma kamatsukae.* Yamaguti (1938) described *Asymphylodora japonica,* a species from *Cyprinus carpio* which had been identified by Nagano (1930) as *A. tincae.* In his report, Yamaguti (1938) stated that the asexual generations of this species occur in *Bulimus striatulus japonicus* and that the tailless cercariae encyst in the rediae. He emended the diagnosis of *A. macrostoma* and reported that larvae of this species encyst in the peribuccal tissue or gill arches of *Chaenogobius macrostomus, Gnathopogon elongatus caerulescens,* and *Cobitus biwae.* Serkova and Bykhovskii (1940) reported both redial and sexually mature stages of a species of *Asymphylodora* in *Bithynia tentaculata* collected near Leningrad, Russia. The worms were distinguished from previously described species by a tabular comparison of morphological features. Although the authors stated that only experimental studies under natural conditions, *i.e.,* the infection of fishes with young trematodes, will permit definite systematic disposition of the specimens, they were described provisionally as a new species, *Asymphylodora progenetica.*

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Szidat (1943) considered the material of Witenberg and Eckmann to consist of two species, one of which he identified as *A. imitans* (Mühling, 1898) and the other he described as a new species, *Asymphylodora carpiae.* The latter species was regarded as identical with *A. tincae kubanicum* Isaichikov, 1923, and if this is true, *A. carpiae* is a synonym of *A. kubanicum.* Szidat distinguished between *A. tincae, A. exspinosa, A. ferruginosa, A. carpiae* and *A. demeli;* he attempted to assign species of *Asymphylodora* to particular hosts, *e.g., A. tincae* occurs apparently in a single host species, *T. tinca,* although other species are not so restricted. For the Asiatic species, *A. macrostoma* Ozaki, 1925 and *A. indica* Srivastava, 1936, Szidat erected a new genus, *Parasymphylodora,* which was suppressed by Skrjabin (1955). Szidat also erected a new genus, *Paleorchis,* for the species with two testes, *i.e., A. diplorchis* Yamaguti, 1936 and two species which he described as new, *viz., P. incognitus* from the intestine of *Leuciscus rutilus* and *P. unicus* from the intestine of *Blicca björkna.* The three genera, *Asymphylodora, Parasymphylodora* and *Paleorchis* were included in a new subfamily, Asymphylodorinae. This subfamily was assigned to the family Lecithodendriidae Odhner, 1910, together with the subfamilies Lecithodendriinae Looss, 1902; Pleurogenetinae Looss, 1899; Haploporinae Looss, 1902; Monorchinae Odhner, 1911; Proctotreminae Odhner, 1911; and Zoogoninae Odhner, 1911. Szidat (1943) stated (p. 70), "Unter Hinweis auf schon früher (1926) erhobene Befunde wird dargelegt, dass die genannten Trematodengruppen lediglich als Unterfamilien der grossen Trematodenfamilie Lecithodendriidae aufzufassen sind, womit ein neuer Beweis für die von dem Autor vertretene Auffassung einer *Parallelentwicklung der Trematoden mit ihren Wirten im Laufe der Phylogenie* erbracht wird und die von W. EICHLER aufgestellten Korrelationsregeln in der Stammesentwicklung von Wirten und Parasiten, die sog. FAHRENHOLZsche und die SZIDATsche Regel erneut bestätigt werden konnten."

Other species, subsequently described as new, include *Asymphylodora atherinopsidis* from the "jack smelt" *Atherinopsis californiensis* taken at Stinson's Beach, California by Annereaux (1947); *Asymphylodora markewitschi* from *Carassius carassius* taken in Russia by Kulakowskaja (1947); *Asymphylodora pontica* from *Neogobius melanostomus* taken in Odessa Bay by Tschernyschenko (1949); *Asymphylodora kedarai* from *Punctius sophora* taken in India by Srivastava (1951); *Asymphylodora macracetabulum* from *Misgurnus anguillicaudatus* taken in Russia by Belous (1954); and *Asymphylodora dollfusi*, a progenetic metacercaria from *Bithynia leachi* taken in the north of France by Biguet, Deblock and Capron (1956). It should be noted that Markewitsch (1951) predicated the identity of *A. dneproviana* and *A. imitans* (Mühling, 1898) and Skrjabin (1955; p. 408) confirmed the opinion. Certain of the species of *Asymphylodora* are described only briefly and others, *e.g.*, *A. atherinopsidis*, which was described from a single worm, are based on a small number of specimens. Which of the twenty named species are actually valid remains to be determined.

Life History and Larval Stages

The life-cycle of members of the genus *Asymphylodora* has been the subject of much controversy. It presents many interesting features and has been under investigation for more than a century. Von Baer (1827) described *Cercaria paludinae impurae* and the species described by de Filippi (1854) from the same host under the name *Distoma paludinae impurae* may be identical with it. It is impossible, however, to make positive determinations on the basis of those early accounts. In the species described as *D. paludinae impurae*, de Filippi reported the finding of a large number of rediae, which produced the distomes directly. These rediae had very short, dilated intestines and no lateral processes of the body wall. The young rediae were motile and moved in a lumbricid manner, while the older ones were inert and contained active distomes, about 0.40 mm. in length, which strongly resembled *Distoma luteum* von Baer, 1826 from *Paludina vivipara*. In this paper, de Filippi reported daughter rediae in a mother generation. He predicated that the production of distomes in a redia is not to be interpreted as an example of simplified development. He stated that these distomes differ from cercariae only in the absence of a tail; he observed cystogenous cells under the body wall, but noted that encystment occurs only outside of the body of the *Paludina*. In a footnote, he described distomes found in *Paludina vivipara* from the Lac de Varése, which measured up to 2 mm. in length and in which the sexual organs were present as rudiments. The worms had no fixed location, but moved about and were found on the mantle. De Filippi expressed the belief that they had come from some other animal and had merely taken refuge in *P. vivipara*. He stated that they were quite distinct from the cercariae which are normal parasites of these mollusks. This large species may be identical with the one from *P. vivipara* described by Swammerdam in the Biblia Naturae (Boerhaave edit. 1752, p. 75, Table IX, Figs. 7, 8) and by von Baer (1826) as *Distoma luteum*.

In a second mémoire, de Filippi (1855) described other distomes from mollusks. These observations demonstrated that tailless cercariae occur in the life-cycles of very different trematodes; that they may be produced in either sporocysts or rediae, and that they may be found in snails without the antecedent generation in which they originated. The absence of a tail, then, is no evidence of genetic relationship.

In a third mémoire, de Filippi (1857) reported that *Distoma paludinae impurae* occurs in two forms which he designated *armatum* and *inerme,* respectively, the latter of which was recognized as the larval stage of *Distoma perlatum* von Nordmann. As noted, Looss (1894) gave a detailed description of *D. perlatum.* He identified the two forms *Distoma paludinae impurae armatum* and *Distoma paludinae impurae inerme* as developmental stages of *D. perlatum* and reported that the cercariae develop in *Bithynia tentaculata.* These two larvae had been transferred to *Cercariaeum* by Diesing (1858) and Szidat (1943) suggested that Looss had found only *D. paludinae impurae armatum.* According to Looss, the cercariae may leave the snail in which they were produced and they may encyst either in the same snail or in others in which no redial stages are present. According to Looss, Wagener (1857) had observed and "hübsch gebildet" this species, but had assigned it erroneously to *Cercaria lymnaei auricularis* de Filippi, 1854. Lühe (1909) accepted the statement of Looss that *Cercariaeum paludinae impurae* from *Bithynia tentaculata* is a larval stage of *Asymphylodora tincae.* He wrote (p. 93) "Die Cercarie, *Cercariaeum paludinae impurae* (Fil.), (schwanzlos, 0.4 mm. lang) entwickelt sich in *Bithynia tentaculata* (L.) in Redien, deren dicker, sackförmiger Darm ungefähr bis zur Grenze der beiden ersten Drittel der Körperlänge reicht (vgl. Fig. 186). Ein Hilfswirt dürfte bei der Schwanzlosigkeit der Cercarie fehlen." In this work Lühe erected the group, Cercariae, with two genera, *Cercariaeum* and *Leucochloridium,* to contain tailless larvae. Members of *Cercariaeum* develop in rediae or unbranched sporocysts and do not encyst. In it he included *C. limnaei obscuri* Ercolani, 1881 and *C. planorbis carinati* de Filippi, 1857 which develop in rediae, together with *C. lymnaei auricularis* de Filippi, 1854 and *C. ancyli lacustris* Diesing, 1855 which develop in sporocysts. Members of *Leucochloridium* develop in branched sporocysts. Sewell (1922) divided the Cercariaea into three sections; *Leucochloridium* was retained but *Cercariaeum* was split into two parts, the "Mutabile Group" which develop in rediae and was named for *C. mutabile* Cort, 1918 and the "Helicis Group" which develop in unbranched sporocysts and was named for *C. helicis* Meckel, 1846. Meanwhile, Fuhrmann found redial and cercarial stages in *Limnea auricularis* var. *ampla* taken in the region of Neuchâtel, Switzerland which he (1916) described as *Cercariaeum squamosum.* The rediae were 2.2 to 3.5 mm. long and the cercariae 0.36 mm. long. The cercariae were covered, except for the ventral area between the suckers, with cuticular scales which were similar to those of species of *Asymphylodora.* Fuhrmann postulated that *C. squamosum,* rather than *C. paludinae impurae* as stated by Lühe (1909), is a larval stage of *A. tincae.*

Dubois (1929) described the redial and cercarial stages of *Cercariaeum squamosum* and reported the species from *Lymnaea limosa, Lymnaea stagnalis* and *Planorbis carinatus.* The rediae were described as simple, saccate structures and Dubois noted such variability in dimensions of body and suckers of cercariae from different individuals of the same species, that he regarded the tailless larvae from all the above named snails as specifically identical. In the Cercariaea, he recognized five groups:

1. Groupe Mutabile. These larvae develop in rediae; they have two testes; the excretory vesicle is tubular and flexible; the flame-cell formula is 2 [(4 + 4 + 4 + 4) + (4 + 4 + 4 + 4)].
2. Groupe Helveticum. These larvae develop in rediae; they have two testes; the excretory vesicle is tubular and flexible; the flame-cell formula is 2 [(3 + 3 + 3) + (3 + 3 + 3)].
3. Groupe Squamosum. These larvae develop in rediae; they have a single testis; the excretory vesicle is small, pyriform or rounded; the flame-cell formula is 2 [(4 + 4 + 4 + 4) + (4 + 4 + 4 + 4)].
4. Groupe Helicis. These larvae develop in sporocysts; number of testes and form of excretory system uncertain.
5. Groupe Leucochloridium. These larvae develop in sporocysts; number of testes and form of excretory system uncertain.

Dubois' observations upheld the morphological agreement, noted by Fuhrmann, between *C. squamosum* and *A. tincae* and accordingly he recognized the identity of the two forms. In the Groupe Squamosum he included *C. paludinae impurae inerme* together with *C. planorbis carinati* and *C. limnaei obscuri*. According to Dubois, *C. paludinae impurae armatum* has a small stylet, two testes, a tubular excretory vesicle, and since it has a flame-cell formula of 2 [(3 + 3 + 3) + (3 + 3 + 3)], it was included in the Groupe Helveticum.

Wesenberg-Lund (1934) described tailless larvae from *Bithynia tentaculata* which he identified as *C. paludinae impurae*. In general morphology these larvae agreed with those from the same host which were described by Dubois (1929) as *Cercariaeum helveticum* I and which were included together with *C. paludinae impurae armatum* to form the Groupe Helveticum. Wesenberg-Lund did not observe a stylet, but otherwise the two forms were very similar. Redial stages and encysted metacercariae were found in the same snail. The cyst walls were thin and membranous. Wesenberg-Lund confirmed the observations of Wunder (1924) that the larvae emerge from the spiracle of the infected snail, pass over the head and proceed along the tentacles, which may appear fluffy in their entire length, with as many as fifty worms on each, or the larvae may accumulate at the tips. The cercariae are always attached by their ventral suckers, with the anterior and posterior portions elevated. If the tentacles come in contact with other snails, the larvae may transfer to the new hosts where they later encyst, usually near the heart or rectum. The author agreed with Dubois that the *inerme* and *armatum* forms of de Filippi are distinct species, but no attempt was made to relate either of them to an adult form. Wesenberg-Lund identified other tailless larvae from *Lymnaea auricularia* as *Cercariaeum lymnaei auricularis* de Filippi, 1854 and his description of these specimens agrees closely with that of *C. squamosum* from the same host as given by Fuhrmann (1916) and Dubois (1929). The two forms are obviously related, but their relation to *A. tincae* is undetermined.

As noted, Serkova and Bykhovskii (1940) described *Asymphylodora progenetica* from the liver and other organs of *Bithynia tentaculata* in Russia. They reported that more than 50 per cent of the mollusks were infected, each with one to seven worms; about 60 per cent of the worms were juveniles, the others sexually mature with eggs in the uterus. None of these worms was encysted. In addition to the juvenile and mature worms, some 15 to 20 per cent of the snails harbored rediae which were presumed to belong to the same species. Each redia contained from one to eight tailless cercariae. Three sets of experiments were performed. In the first, uninfected specimens of *B. tentaculata* were placed in aquaria with infected ones. At the end of one month, both juvenile and gravid worms were found in the previously uninfected snails. The worms were always free; none was encysted. No rediae were found in the previously uninfected individuals. There was no statement concerning the number of snails used in the experiment nor of the criteria used to determine whether or not the snails were infected. In a second experiment, fifteen uninfected specimens of *B. tentaculata* were placed in each of three aquaria and eggs of *A. progenetica* were added. At the end of twenty days the following results were obtained: in Aquarium I, there was one snail which contained an embryonic mass, presumed to be of *A. progenetica,* and the others were not infected; in Aquarium II, one snail contained eight mature specimens of *A. progenetica* with eggs in their uteri and four dead worms, while the other snails were not infected; in Aquarium III, none of the snails was infected. From these results, the authors concluded that the life-cycle of *A. progenetica* can be completed in *B. tentaculata,* and that the development from eggs to mature adults can take place in twenty days. In the third experiment, pieces of *B. tentaculata* were fed to cyprinid minnows, *Rutilus rutilus* and *Carassius carassius.* In *R. rutilis,* the worms persisted for six days but were absent after fifteen days; in *C. carassius,* the worms were present after twenty-four hours, but not at the end of three days. The experiments reported by Serkova and Bykhovskii are not at all convincing. In the absence of information concerning controls, the results can not be evaluated properly. However, the belief that *B. tentaculata* harbors a single species, and that the rediae and cercariae were larval stages of the species whose juvenile and sexually mature stages were described as *A. progenetica,* was not adequately supported. The results of the attempt to infect *B. tentaculata* discredit the stated conclusions. The failure to obtain rediae suggests the absence of experimental infection. The finding of an embryonic mass in one snail is equivocal, and the snail with eight gravid worms was certainly infected at the time of exposure.

Szidat (1943) confirmed earlier observations of Dubois (1929) and Wesenberg-Lund (1934). He reported that the adult stage of *C. paludinae impurae armatum* occurs in *Leuciscus rutilus* and for it he erected a new genus, *Paleorchis.* He noted that the first three groups of Dubois are closely related, whereas the fourth and fifth groups, which develop in terrestrial snails, are members of a different family, Harmostomidae (= Brachylaemidae). Szidat declared that the larvae of *Asymphylodora* certainly belong in the Squamosum group of Dubois. He recognized *C. limnaei auricularis* de Filippi, 1854 and *C. squamosum* Fuhrmann, 1916 as distinct species, and the former was identified as the larva of *A. tincae.* Provisionally, the latter species was referred to *A. imitans* (Mühling, 1898). Szidat considered the probability that *C. planorbis carinati* de Filippi, 1857; *C. limnaei obscuri* Ercolani, 1881; and *C. squamosum* Fuhrmann, 1916 are identical with *C. paludinae impurae inerme* de Filippi, 1854.

The five larval groups of Dubois have now been correlated with their adult stages. Wallace (1941) reported that *C. mutabile* Cort, 1918 is the larva of a species of *Triganodistomum* Simer, 1929. The Helveticum group contains the larvae with stylets, two testes, which according to Wunder (1924) leave their primary hosts and encyst in secondary hosts. They were identified by Szidat (1943) as developmental stages of species of *Paleorchis*. Members of the Squamosum group are larvae of *Asymphylodora*. Thomas (1959) transferred *Triganodistomum* to the Monorchiidae and thus, members of the three groups belong in the family Monorchiidae or in the subfamily Monorchiinae if the proposal of Szidat is accepted and the monorchids are regarded as a subfamily in the expanded family Lecithodendriidae.

The identity of corresponding stages of species in the genus *Asymphylodora* is yet undetermined. Deblock, Capron and Biguet (1957) reported specimens of *Asymphylodora* from the intestine of *T. tinca* taken in the north of France. These worms differed from descriptions of *A. tincae* as given by earlier authors, including Szidat (1943) who associated *A. tincae* exclusively with *A. tinca*. The worms described by the French authors were differentiated from previous descriptions of *A. tincae* on the basis of three features, *viz.*, the presence of a short common sperm duct before the seminal vesicle, the extension of ventrolateral pores to converge and meet behind the acetabulum, and the absence of spines on the ventral area between the suckers. The worms were designated as a new variety, *Asymphylodora tincae mediaglabra*. The question was raised if the larval form of the classically described form of *A. tincae* is not *C. paludinae impurae inerme* as postulated originally by de Filippi (1857).

EXPERIMENTS

Stunkard (1955) reported the finding in the summer of 1954 of both juvenile and sexually mature trematodes in the fresh-water pectinibranchiate snail, *Amnicola limosa,* collected from ponds in the vicinity of Woods Hole, Massachusetts. About one-fourth of the fully grown snails were infected. From one to nine unencysted worms, identified as members of the genus *Asymphylodora,* were found in an infected snail. As a rule, when several specimens were present only one or two contained eggs, but as many as five gravid worms were found in a single snail. The presence of worms of different sizes and degrees of development, from very young to gravid specimens, indicated successive repeated infections. When removed to a glass slide, juvenile specimens frequently encysted but encystment of sexually mature worms was not observed. The cyst walls were thin and membranous. No sporocyst or redial stages were found at the time and it appeared that the asexual generations of the worms occur in some species other than *A. limosa*.

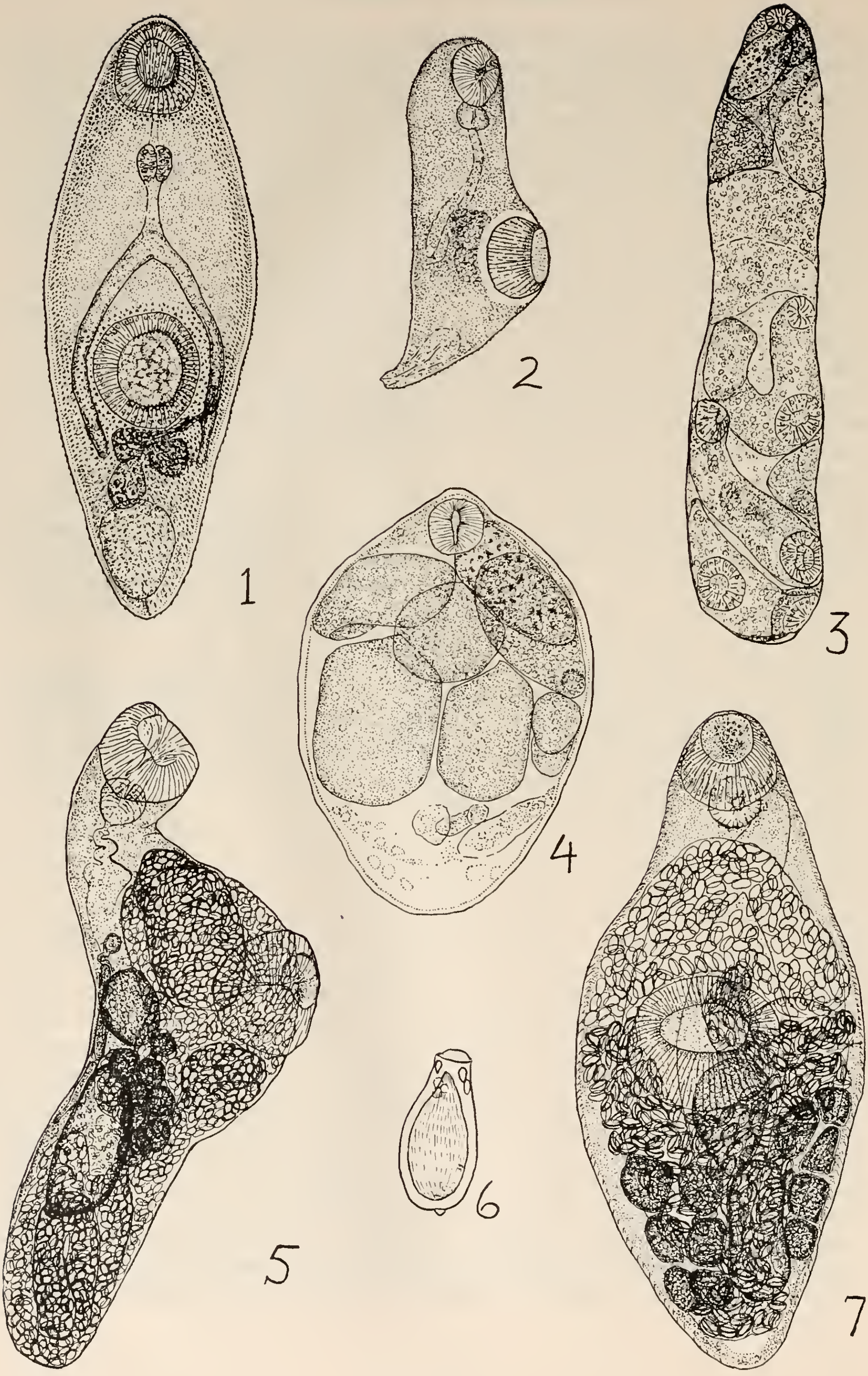
The worms were found, but only rarely and in small numbers, in the intestines of fishes from the same ponds. They were taken from yellow perch (*Perca flavescens*), fresh-water killifish (*Fundulus diaphanus*), small-mouth bass (*Micropterus dolomieu*) and bluegill sunfish (*Lepomis macrochirus*). Whether the fishes obtained the worms by eating snails or by eating small fishes which had eaten snails is unknown. Trematodes from ingested fishes may persist for some time in the intestines of predatory animals and parasites of the prey may easily be mistaken for parasites of the predator. The eggs of the parasite contained miracidia and those in worms from snails were identical in appearance with those in worms taken from the intestine of fishes. The ponds had been stocked at various times by the Division of Fisheries and Game of the State of Massachusetts.

Subsequently, redial and cercarial stages of this parasite have been found in natural infections of *A. limosa;* from one to three per cent of the snails in different collections. In addition, sporocysts and rediae have been recovered from experimentally infected, laboratory-reared snails. *Amnicola limosa* is a dioecious species; eggs are laid singly and young specimens are present in numbers in laboratory cultures. The snails are temperamental feeders, living principally on diatoms, and it is difficult to maintain them for long periods, especially when infected. Cultures of diatoms were provided by Dr. Luigi Provasoli of the Haskins Laboratories in New York, for which grateful acknowledgment is made.

Eggs passed by worms taken from snails were maintained in pond water for one to six days to insure embryonation and then fed to young, laboratory-reared *A. limosa.* Each snail was placed in a stender dish, 5 cm. in diameter, together with a number of eggs. The next day the snail was removed to a larger container for more favorable conditions. Dissection of these snails, beginning after one week, disclosed sporocysts and rediae indistinguishable from those of natural infections. Whether or not there is a daughter sporocyst generation was not determined.

Cercariae removed from the tentacles of a snail were placed in a small stender dish, 3 cm. in diameter, with a small uninfected, laboratory-raised *A. amnicola.* The following day they had disappeared and the snail was dissected. About one-half of the cercariae had encysted. This finding is difficult to interpret since very few cysts have been found in snails from ponds, although often one-third or more of them contained unencysted juvenile or gravid worms.

If a snail harbors the asexual generations, the tailless cercariae emerge and migrate to the tentacles where they cling in the manner described by Wunder (1924) and Wesenberg-Lund (1934). The worms adhere by the acetabulum, with the ends of the body extended and elevated. The larvae may remain attached at any location on the tentacles and sometimes accumulate near the tips. When many cercariae are attached, the tentacles may manifest a feathery appearance. If such an infected snail makes contact with an uninfected one, the cercariae may transfer to the tentacles or body of the uninfected specimen. In the new host, they often encyst in thin membranous envelopes that are not reinforced with a cyst wall deposited by the host. If cercariae are removed from tentacles and placed in a small stender with the same snail, they soon regain their position on the tentacles. If placed in a dish with an uninfected snail, they attach and may migrate to the tentacles, but they do not remain there; instead, they move about and in most instances enter the tissues. In snails that do not harbor asexual generations, the worms may be recently encysted, they may be unencysted juveniles of older infections, or unencysted gravid specimens. Such worms often contain hundreds of eggs and those in the terminal part of the uterus contain fully formed miracidia. No rediae have been found in snails that harbored juvenile or sexually mature worms, and no juvenile or mature worms in snails that harbored the asexual generations. It appears that the cercariae leave an infected snail and enter only snails that do not harbor asexual infections. Since several worms, often two to four of them gravid, may occur in a snail, the presence of one worm does not confer complete immunity and inhibit the entrance of others. Juvenile worms may encyst or remain unencysted since such specimens retain their cystoge-



Figures 1–7.

nous glands for some time and can encyst if removed from their hosts. The worms grow in their cysts since they increase somewhat in size, but major growth and maturity take place in unencysted specimens. It appears if the metacercariae encyst, that later they emerge because all older and gravid worms were free in the tissues of the snail.

DESCRIPTIONS

The adult (Figs. 5, 7)

The worms are fusiform, wider near the middle and narrower at the ends. The preacetabular portion of the body is almost cylindrical and since the acetabulum is large and often protruding, fixed and stained specimens are frequently oriented on the side (Figs. 2, 5), with the dorsoventral measurement greater than the width. In cercarial and juvenile specimens the acetabulum is in the posterior half of the body but with sexual maturity and the accumulation of eggs in the uterus, the acetabulum is shifted relatively farther forward and may come to lie in the anterior half of the body. The worms continue to grow after sexual maturity is attained. Specimens from fishes as a rule are somewhat larger than those from snails. Gravid worms from snails when fixed, stained and mounted measure from 0.28 to 0.58 mm. in length and 0.14 to 0.24 mm. in width; corresponding measurements of worms from fishes are 0.40 to 0.71 mm. and 0.18 to 0.25 mm. The acetabulum measures 0.09 to 0.13 mm. in diameter.

The anterior end of the body in juvenile specimens bears a number of protrusible papillae, each provided with a seta, and these papillae are sometimes visible in gravid specimens. Other papillae are present at the rim of the acetabulum. The cuticula has a characteristic spination. Over much of the anterior half of the body the spines are slender, sharp, distinctly separated and 0.006 to 0.008 mm. long. On the ventral side, the median area between the suckers lacks spines and on the dorsal side in the corresponding area the spines are reduced in size and number. On the posterior part of the body the spines are broader, more scale-like in appearance and less numerous. Around the openings of the suckers there are six to seven rows of very small spines. They are closely set, triangular in shape and manifest a serrate appearance. From these areas, the spines gradually change in size and shape to conform with the aciculate and scale-like types on the adjacent areas. The parenchyma of the body, especially in the anterior portion, contains a large number of glandular cells; the ducts of many of them lead forward to open near the anterior end of the body. Other glandular cells open

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The figures, except that of the egg, were made from fixed and stained specimens.

FIGURE 1. Metacercaria, removed from cyst; much flattened for study of the excretory system; ventral view, 0.43 mm. long.

FIGURE 2. Cercaria, removed from a redia; lateral view; 0.19 mm. long.

FIGURE 3. Redia; natural infection; a large specimen; 0.9 mm. long.

FIGURE 4. Redia; one of four, from experimental infection of a laboratory-raised snail; 0.19 mm. long.

FIGURE 5. Adult; lateral view; one of the largest specimens; from *Perca flavescens;* 0.70 mm. long.

FIGURE 6. Egg, extruded from a gravid worm after removal from a snail, examined in water; 0.027 mm. long.

FIGURE 7. Adult; ventral view; somewhat compressed; from *Amnicola limosa;* 0.49 mm. long.

on the ventral side, especially in the area between the suckers, but rows of pores as reported by Looss (1894) and by Deblock, Capron and Biguet (1957) were not apparent. Such a linear arrangement would probably be produced only when that portion of the body is extended, otherwise the alignment would be disturbed.

The oral sucker is 0.06 to 0.095 mm. in diameter. The mouth is subterminal; there is a short prepharynx and the pharynx measures 0.036 to 0.044 mm. in diameter. The esophagus is long, the length dependent on the degree of extension of the preacetabular portion of the body, and the bifurcation of the digestive tract is immediately in front of the acetabulum. The ceca extend posteriad on the dorsal side of the body and end blindly at the level of the posterior part of the testis. The worms in the snails feed on tissues, as the digestive ceca contained cells, nuclei and cellular debris that could have come only from the molluscan host.

The excretory pore is terminal; the vesicle is small, lined with high epithelial cells. The collecting ducts pass forward to the level of the pharynx where they turn backward, and shortly anterior to the acetabulum they divide into anterior and posterior branches. Each branch has four groups of four flame-cells and the formula is $2\ [(4+4+4+4)+(4+4+4+4)]$, which is characteristic for members of the Squamosum group of cercariae. These observations were made on juvenile specimens (Fig. 1) since in gravid worms the development of the reproductive organs and the accumulation of eggs in the uterus make it impossible to work out the flame-cell pattern.

There is a single testis although the presence of two sperm ducts indicates its dual nature. It is median, located near the posterior end of juvenile and young specimens. As the body becomes congested with eggs in the uterine coils, the testis is shifted more dorsad and forward; indeed the post-testicular region may be as long as the testis. The testis is oval, 0.08 to 0.13 mm. in length and 0.06 to 0.10 mm. in width. Two sperm ducts pass forward, one from each side of the testis. In certain specimens they unite immediately before the cirrus sac and in others they unite to form a short duct which leads to the cirrus sac. Within the sac, the duct expands to form a twisted or coiled seminal vesicle which may appear bipartite or tripartite. The seminal vesicle is succeeded by a short prostatic region and then the ejaculatory duct, lined with spines, leads to the common genital pore. The prostatic cells occupy most of the space in the cirrus sac in front of the seminal vesicle. The ejaculatory duct may be everted to form the protruded cirrus. The cirrus sac extends in a curved course from the level of the anterior end of the ovary to the genital pore, situated on the left side at the acetabular level.

The ovary is dextral, immediately anterior to the testis, or the two may overlap. It is oval, somewhat smaller than the testis, and measures 0.06 to 0.11 mm. in length and 0.05 to 0.09 mm. in width. The oviduct arises from a ventral prominence near the posterior end of the ovary and makes a median posterior bend, after which it expands into a small seminal receptacle from which Laurer's canal extends to the dorsal surface of the body; it then receives the common vitelline duct and expands to form the ootype, enclosed in the cells of Mehlis' gland. The initial part of the uterus contains spermatozoa. From the ootype, in young specimens, the uterus passes forward to the level of the anterior end of the acetabulum, then back on the right side to the posterior end of the body and forward on the left side to join the metraterm. But in older, fully gravid individuals, the anterior loop extends forward to the pharynx, coils about in the preacetabular region, filling much of the body between the suckers, and the posterior loop also winds about filling and expanding this portion of the body. In general, the uterine coils lie below the gonads and vitellaria and their course can be followed by the color of the eggs which are light in the initial part of the uterus and become darker as they proceed. The metraterm is a thicker-walled, terminal part of the uterus, about one-third as long as the cirrus sac, and the lumen is lined with cuticula which bears spines, similar to those which line the ejaculatory duct. There are nine vitelline follicles on each side of the body; typically there are five in a median, longitudinal row and four in a lateral row but contraction of the body muscles and pressure from other organs may disturb this arrangement. The follicles measure 0.033 to 0.043 mm. in diameter and are situated in the region between the acetabulum and the posterior end of the testis. Large collecting ducts from the follicles of each side pass mediad on the dorsal side of the body to form a vitelline receptacle, from which the common duct leads anteriad and ventrad to join the oviduct. The eggs (Fig. 6) are ovate, slightly narrower at the opercular end and somewhat flattened on one side. There is a thickened projection or knob asymmetrically situated near the antopercular end of the egg. The young eggs are almost colorless and the cells within stain readily; mature eggs have thick, yellow shells and contain a miracidium. The eggs measure 0.025 to 0.029 mm. in length and 0.013 to 0.016 mm. in width.

The sporocyst

No sporocysts of natural infection were found. In a snail exposed on July 3 and sacrificed on July 12, there was a sporocyst which measured 0.07 by 0.52 mm. It contained only germinal cells. Another sporocyst found on July 25 in a snail exposed on July 10 was slightly larger and in addition to germinal cells it contained three small germinal masses, embryos of the next generation.

The redia (Figs. 3, 4)

The rediae are simple sausage-shaped structures, without collar or feet, typically cylindrical although often attenuated at one or both ends. The body wall contains both circular and longitudinal muscles, so the rediae are slender when elongated and plump when retracted. In size they measure up to 1.00 mm. in length and 0.2 mm. in width, although most of them are only about one-half this size. There is a birth-pore situated near the pharynx. The pharynx has a diameter of 0.035 to 0.040 mm.; the gut has a short neck-like portion and the intestine is saccate. In young, small rediae it may extend one-third of the length of the body but it does not increase appreciably in size, and in older, mature specimens it occupies only a small fraction of the body cavity. Figure 4 shows one of four rediae found in a laboratory-raised snail exposed on July 14, 1955, and dissected on the following September 7th. The rediae were all approximately the same size. The one shown in the figure measures 0.19 by 0.13 mm. and the pharynx is 0.030 mm. in diameter.

The cercaria (*Fig. 2*)

The cercariae grow to large size in the rediae. Specimens removed from rediae or taken from tentacles of snails measure from 0.16 to 0.32 mm. in length and 0.06 to 0.12 mm. in width. The oral sucker is 0.05 to 0.06 mm.; the acetabulum 0.06 to 0.07 mm.; and the pharynx 0.033 to 0.035 mm. in diameter. The reproductive organs are represented by a lobed mass of germinal cells situated dorsal and perhaps slightly posterior to the acetabulum. The excretory system was not fully worked out in cercariae, but apparently it is completed in the cercarial stage and so far as determined, is identical with that found in juvenile worms.

The metacercaria (*Fig. 1*)

Cercariae from the tentacles of a snail were placed in a small stender dish with a small, laboratory-reared *A. limosa*. The following day they had disappeared and the snail was dissected. About one-half of the cercariae had encysted. The cysts measured 0.19 to 0.22 mm. in diameter; they were very thin-walled and ruptured easily under pressure of a coverglass. A few encysted specimens have been found in naturally infected snails, but almost all the metacercariae from natural infections were unencysted. On a few occasions, juveniles removed from naturally infected *A. limosa* encysted when removed to a slide for observation. The specimen shown in Figure 1 was removed from a cyst; it is hardly more than a cercaria since it had been removed from the tentacle of a snail the day before. It was very much flattened to study the excretory system. The unencysted metacercariae feed and grow; some of the juveniles were as large as worms with eggs in the uterus. Large numbers of dead, gravid worms have been found in naturally infected snails.

Except for the single specimen from the marine silverside, *Atherinopsis californiensis,* described by Annereaux (1947) as *Asymphylodora atherinopsidis* n. sp., the genus *Asymphylodora* has not been reported previously from North America. Mature specimens from *A. limosa* are smaller than those of either *A. progenetica* or *A. dollfusi*. The suckers are smaller, but the gonads and eggs are larger. There are differences also in the number of vitelline follicles. Description of the several stages of the species found in *A. limosa* taken from ponds in the Woods Hole region indicates that the specimens can not be assigned to any known species, and they are described as a new species, *Asymphylodora amnicolae*.

Discussion

In the paper describing *Distoma paludinae impurae,* de Filippi (1854) made the first distinction between sporocysts and rediae. He noted that *Redia gracilis,* described by him (1837), was a developmental stage of an amphistome and that similar stages occur in other species. Accordingly, he declared that the term, Redia, is not a generic name but like Cercaria O. F. Müller, 1773, is merely a stage in the life-cycle of certain trematodes. He characterized sporocysts as simple, membranous sacs without internal organization, whereas rediae are provided with a mouth, muscular pharynx, and intestine. In certain species, the body of the redia may form temporary lateral processes. It was common knowledge that cercariae could be produced in either sporocysts or rediae and de Filippi reported daughter rediae in a mother generation. The term *"méta-cercaire"* was proposed by Dollfus (1913) to designate the immediate postcercarial stage, whether encysted or not, although in later publications he wrote the name as a single word, métacercaire. The designation of this stage is a contribution which facilitates description and discussion of life-cycles among digenetic trematodes.

Sexual maturity and production of eggs by metacercariae had been known since the report by von Siebold (1835) of eggs emerging from metacercariae found in *Astacus astacus.* For sexual maturity of animals which had not yet attained adult condition, Giard (1887) proposed the term "progenèse" and Dollfus (1924) applied the designation to gravid metacercariae of digenetic trematodes. Sinitsin (1905) reported sexually mature specimens of *Pleurogenes medians* encysted in aquatic larvae of *Agrion* sp. taken in the vicinity of Warsaw. Joyeux (1923) found gravid specimens of *Ratzia parva* in cysts from *Rana esculenta* in North Africa and obtained swimming miracidia from the eggs. Mathias (1924) obtained adults of *Pleurogenes medians* in *Rana temporaria* and *Hyla arborea* after feeding encysted metacercariae from *Gammarus pulex.* Many of the metacercariae contained eggs.

Dollfus (1927) described progenetic metacercariae of *Dinurus tornatus* (Rudolphi, 1819) and wrote (p. 55), "La découverte de cette forme permet cependant de se poser une fois de plus la question de savoir s'il peut exister, chez les trématodes digénétiques, des cycles abrégés par suppression de l'hôte définitif; l'on n'a pas, toutefois, démontré que les oeufs formés par les métacercaires progénétiques soient susceptibles de donner des miracidia infectants pour le mollusque premier hôte. Il semble que l'expérience seule pourra fournir la solution de ce problème si intéressant pour la biologie générale." Fuhrmann (1928) reported progenetic metacercariae of *Ratzia parva* in cysts from the muscles of *Rana temporaria,* and Dollfus (1929) again raised the question, "Existe-t-il des cycles évolutifs abrégés chez les trématodes digénétiques? Le cas de *Ratzia parva* (Stossich, 1904)." He stated (p. 203), "La métacercaire est progénétique, elle donne des oeufs contenant un miracidium. L'on n'a pas encore pu établir si ce miracidium renferme des éléments germinaux et est infestant pour le mollusque premier hôte, mais le seul fait que ce miracidium existe et nage activement dans l'eau après éclosion de l'oeuf permet de se demander s'il n'existerait pas, chez *Ratzia* outre un cycle évolutif complet comprenant un adult vrai dans l'hôte définitif, un cycle abrégé dans lequel la métacercaire jouerait le rôle d'adult et le deuxieme hôte intermédiare le rôle d'hôte définitif." Reproduction in progenetic metacercariae of *R. parva* was discussed by Joyeux, Noyer and Baer (1930). Dollfus (1932) described progenetic metacercariae from *Planorbis planorbis* and reported (p. 412), "Nous signalons le premier cas connu d'une métacercaire progénétique chez un mollusque d'eau douce. Le même planorbe héberge des sporocysts, les cercaires, les métacercaires enkystées et les métacercaires libres, vraisemblablement sorties de leur kyste." Since the larvae attained sexual maturity in their cysts and contained both spermatozoa and developing eggs, Dollfus admitted the possibility of self-fertilization.

Alice Buttner (1950a) stated (p. 21), "Les métacercaires progénétiques sont surtout fréquentes chez arthropodes (insectes, crustacés), ainsi que chez les poissons et les batraciens; elles ont été rencontrées également quelquefois chez des mollusques." She reported for *Plagiorchis brumpti* the first experimental demonstration of an abbreviated life cycle in a digenetic trematode and predicated (p. 25), "La progénèse est, chez les trématodes, un phénomène tantôt constant, tantôt accidentel; dans ce dernier cas, les métacercaires non progénétiques restent susceptibles, comme les progénétiques, de devenir adultes chez un hôte définitif." In a comprehensive study of progenesis among digenetic trematodes, Mlle. Buttner (1950b, 1951) reviewed previous accounts, listed progenetic species in more than a score of genera and a dozen different families, and reported experiments showing constant, obligatory progenesis in *Paralepoderma brumpti* and *Ratzia joyeuxi* and inconstant, facultative progenesis in *Pleurogenes medians.* Other observations were made on identified and unidentified progenetic cercariae and metacercariae.

Biguet, Deblock and Capron (1956) described progenetic metacercariae from *Bithynia leachi* collected in the north of France. Dissection of these mollusks revealed an infection rate of more than 30 per cent, with one to twelve unencysted specimens in each host. The worms manifested varying degrees of maturity and about 10 per cent of the infected snails contained rediae with developing cercariae. Sporocysts were not observed. Comparison with the description of *A. progenetica* as given by Serkova and Bykhovskii (1940) and other described species disclosed differences which led the authors to designate their specimens as members of a new species, *Asymphylodora dollfusi.* Observations on the developmental cycles of *Asymphylodora tincae* and other digenetic trematodes were reported by Wisniewski (1956).

Observations on abbreviated life-cycles have been recorded also for members of other taxonomic groups. Progenetic metacercariae of hemiurid species were reported from copepods by Dollfus (1954) and by Chabaud and Biguet (1954). Adult specimens of another hemiurid were reported from *Sagitta elegans* by Betty Myers (1956). Crusz (1956) reported progenetic metacercariae of *Cercaria patialensis,* a species described by Soparkar (1924) from *Melanoides tuberculata* and related to *Transversotrema* by Witenberg (1944). Peters (1957) described *Allocreadium neotenicum* n. sp., from sexually mature specimens found in water beetles. Stunkard and Uzmann (1959) reported that sexually mature and gravid worms found in *Mytilus edulis* are specifically identical with *Cercaria milfordensis* Uzmann, 1953 and with *Proctoeces maculatus* (Looss, 1901). Freeman and Llewellyn (1958) described adult worms in the renal organs of the lamellibranch mollusk, *Scrobicularia plana,* taken from the flats of the Thames estuary, at Chalkwell in Essex and Whitstable in Kent. The specimens were identified as *Proctoeces subtenuis* (Linton, 1907), which Stunkard and Uzmann (1959) synonymized with *P. maculatus.*

The extent and incidence of progenetic maturity in larvae of digenetic trematodes have aroused questions in the minds of many investigators since Dollfus posed the problem. After reporting sexual maturity in metacercariae of a species of *Clinostomum,* McIntosh (1935, p. 79) wrote, "Do these records of precocious development throw light on what the adult flukes were like in the ages before the vertebrates came into being, or may the phenomenon be explained on the basis of the similarity of the environment of the metacercaria with that of the habitat of the adult?" Buttner (1951) attempted to evaluate the environmental factors which might affect sexual development and raised the question whether or not progenesis might provide data concerning the origin of parasitism among the trematodes; whether it represents successive stages in the evolution of existing life-cycles or is merely an abridgment or simplification of otherwise more complicated cycles. She was inclined to regard progenesis as the result of a mutation in hereditary factors rather than as the result of environmental influences. Szidat (1956) discussed the causes and meaning of abbreviated life cycles in trematodes and cestodes. He stated that formation of eggs in the first intermediate host would undoubtedly be favorable for preservation of the species and that the addition of intermediate hosts might be explained as steps in the elaboration of a food-chain.

The origin of metagenesis, alternation of sexual and asexual generations, has received the attention of biologists for more than one hundred years. It was first recognized by Steenstrup in coelenterates and parasitic flatworms. Actually, the phenomenon was initiated and perfected in plants. In the early amphibious plants, liverworts and mosses, the sexual gametophyte was dominant and the asexual sporophyte arose as a microscopic and parasitic generation when germinal cells separated from the parent organism and, enclosed in a protective covering, proved capable of developing new gametophytes. It is probable that the development of the sporophytic generation was an adaptation to terrestrial existence. In the course of evolution, as represented by the pteridophytes, both generations were independent with the sporophyte gradually assuming the ascendancy, while in seed-bearing plants the asexual sporophyte became dominant with the sexual generation reduced to a few cells, entirely dependent and parasitic on the asexual one. In plants, the gametophyte is haploid and the sporophyte is diploid, with reduction of chromosomes at sporogenesis. In the Coccidia and other sporozoans, reduction is zygotic, whereas in the trematodes both generations are diploid and reduction is gametic. It appears, therefore, that the location in the life-cycle where the reduction divisions occur is not determinate in the evolution of metagenesis.

Among endoparasites, the parasite perishes with the host, and many species which became successful developed accessory, *i.e.,* asexual types of reproduction and provision for emergence and dispersal of the asexually produced individuals. The introduction of asexual methods was essentially similar in plants and animals. In both they evolved in a parasitic generation. In the protozoans which became cellular or histozoic parasites, sporulation became the characteristic method of reproduction with alternation of generations. Sporulation involves rapid nuclear divisions in a common cytoplasmic matrix, the formation of a plasmodium, and subsequent multiple fission with the production of many new individuals. In the asexual cycle, each cell which results from sporulation is termed a merozoite. Certain of these cells, usually after repeated divisions and the development of physiological maturity, become gametocytes and produce gametes. In the sexual cycle, the fusion of gametes produces a zygote which again undergoes sporulation and the production of sporozoites. Both types of reproduction may take place in the same host or, in other species, alternation of generations and hosts may occur.

The parasitic flatworms were derived from free-living species by gradual and progressive adaptation and present life-cycles are not primitive. The Digenea are distinct in morphology, bionomics and life-cycles from the Monogenea and the origin of double hosts with alternation of generations, involving sexual and asexual types of reproduction, is still an unresolved problem. It is generally agreed that the Digenea have a common ancestry with the Turbellaria. In certain rhabdocoels, asexual reproduction regularly alternates with sexual reproduction. It appears that asexual reproduction is associated with rapid metabolism and a juvenile condition and that sexual maturity is attained with physiological maturity. The turbellarians are primitively carnivorous and certain dalyellioid species, probably related to the ancestors of the Digenea, are parasitic in mollusks and echinoderms. Others are commensals or pseudoparasites. Leuckart maintained repeatedly that the mollusk was the original host of the early digenetic trematodes and his argument was indorsed by Stunkard (1957).

It is accepted that endoparasitism frequently results in the acquisition of accessory, asexual methods of reproduction. If the ciliated larva of the turbellarian, on invasion of a mollusk, were to form a cyst-like structure in which the germinal cells were to separate and develop independently, a very efficient method of reproduction, polyembryony, would have arisen. The repeated dissociation of germinal cells could continue to form daughter sporocysts, but the progeny would tend eventually to develop turbellarian structures, notably a pharynx and simple gut, and a redia-like individual would result. Such a sexually mature stage may be discerned in members of the Aspidogastridae.

Polyembryony in sporocysts and rediae would produce population pressure and individuals would be expelled from the host. The finding of a new host required locomotion and the modification of the posterior portion of the body to form a device for sculling through the water gave a distinct advantage over mere wriggling movements or the successive attachments of suckers. The diversity in types of cercarial tails indicates the many directions in which this adaptation has progressed. The asexually produced individuals, on leaving their former hosts, would be dispersed throughout the neighboring area. Such worms, already pre-adapted for parasitic existence, would invade and become sexually mature in other animals, and in essential respects could be compared with present-day progenetic metacercariae. With the advent of vertebrates, these parasites together with their hosts were eaten; as a result the worms were introduced into new hosts. With establishment in the newly acquired vertebrate hosts, sexual maturity was naturally more and more deferred to worms in the definitive hosts. Meanwhile, the former definitive hosts became secondary intermediate, paratenic or transport hosts, providing for completion of the newly acquired life-cycle. The vertebrates, wider-ranging and longer-lived animals, would facilitate the dispersal and prolong the life of the parasites. After reviewing life cycles of parasitic worms in the different classes, Stunkard (1953) concluded, ". . . it is apparent that developmental cycles have been evolved concomitantly with the parasitic habit, that they are bionomic adaptations to ecological conditions, and that modifications in the course of the life-cycle were introduced accidentally and have been perpetuated because they facilitated dispersal and had survival value for the species." Progenesis occurs in members of many families; its distribution and incidence are too extensive to be explained as a mutation; rather it appears to be a relict, the persistence of an earlier state, which obtained before sexual maturity was so largely deferred to definitive vertebrate hosts.

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STUDIES ON THE PHYSIOLOGICAL VARIATION BETWEEN TROPICAL AND TEMPERATE ZONE FIDDLER CRABS OF THE GENUS UCA. III. THE INFLUENCE OF TEMPERATURE ACCLIMATION ON OXYGEN CONSUMPTION OF WHOLE ORGANISMS[1]

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As organisms extend their distributional limits, they are faced with new environmental stresses. Through the mechanism of natural selection, populations of animals have arisen which show varying degrees of physiological adaptation. Review papers by Prosser (1955) and Bullock (1955) have excellent discussions of many aspects of this subject.

The present series of papers deals with physiological variation between temperate and tropical zone species of fiddler crabs (genus *Uca*). Results of the first paper (Vernberg and Tashian, 1959) demonstrated the marked difference in thermal death limits, especially at low temperatures, of latitudinally isolated populations of fiddler crabs. The 50% mortality level reached after 30–40 minutes at 7° C. for *Uca rapax* was characteristic of the other fiddler crabs studied in Jamaica, The West Indies, while temperate zone species from North Carolina lived for weeks at this temperature. Acclimation to reduced temperature (15° C.) had little effect on the response of tropical species but greatly increased the survival time of temperate zone forms. The second paper (Vernberg, 1959) dealt with the oxygen consumption of fiddler crabs as influenced by temperature, season, size and starvation. The seasonal variation in rates of oxygen consumption observed in *U. pugnax* from North Carolina, but not in the similar sized crab, *U. rapax* from Jamaica, suggested that temperate zone crabs possessed a labile respiratory mechanism while the response of tropical species is fixed within narrow limits.

The importance of temperature acclimation studies in understanding the metabolism of latitudinally isolated populations has been stressed for some time. Therefore the present investigation was undertaken to study in more detail the role of acclimation in determining metabolic differences between tropical and temperate zone species. Since this study began, papers on crab respiration by Démeusy (1957), Roberts (1957) and Teal (1959) have further emphasized the need for additional work on this problem.

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[2] A portion of this work was done while serving as a John Simon Guggenheim Memorial Fellow.

MATERIALS AND METHODS

In the present paper two species of *Uca* were selected, *Uca pugnax* (Smith) from North Carolina and *Uca rapax* (Smith) from Jamaica and Florida. These particular species were chosen for a number of reasons: both species are closely related; until recently (Tashian and Vernberg, 1958), both species were considered races belonging to one species, the southern form being *U. pugnax rapax* and the northern race *U. pugnax pugnax*. Populations of these two species are found on the coast from Massachusetts to Brazil with a zone of overlap along the northeast coast of Florida. Thus they represent an excellent continuous series for a comparative study. Both species not only are available in great numbers, but they are easily maintained under laboratory conditions. Results of acclimation studies could be correlated with detailed metabolic data already available on these two species (Vernberg, 1959).

Experimental studies on fiddler crabs from North Carolina and Florida were conducted either at the Duke University Marine Laboratory or at Duke University, and the tropical species was studied at the University College of the West Indies, Jamaica.

The method of determining oxygen consumption, as well as the procedure for the laboratory care of animals, was described in a preceding paper (Vernberg, 1959). All results are expressed as cu. mm. oxygen consumed/minute/gram of animal (wet weight). Preliminary studies showed that the time interval necessary for thermal equilibration and for the rate of oxygen uptake to reach a somewhat steady level varied inversely with temperature. Only oxygen consumption data which were relatively stable over a period of time were used. An attempt was made to minimize variation due to rhythmic daily fluctuations by making an equal number of determinations in the afternoon and the morning. No attempt was made to correct for possible tidal influence on metabolism. Although the determinations were made at different times of the year, the recent thermal history of animals from North Carolina and Jamaica was the same in that their habitat and laboratory temperatures were alike. Work at Beaufort extended from June to September, while studies in Jamaica were from October, 1957, to April, 1958. Animals from Florida were collected in October and November and maintained in the laboratory until needed.

Animals subjected to 15° C. for various periods of time are referred to as cold-acclimated or "cold" animals, while animals maintained at room temperature (22–27°) will be called warm-acclimated or "room" animals.

RESULTS

Uca pugnax from Beaufort, North Carolina

The metabolic response of 21 warm-acclimated *U. pugnax* was determined at 27° C. and then these animals were kept at 15° for 21 days. Throughout the period of exposure to reduced temperature the rate of oxygen consumption was re-determined at 27° C. at specific intervals of time. Another group of animals, which were maintained in a starved condition at room temperature, were used as a control. Thus the per cent change in metabolic rate shown in Figure 1 represents shifts in rate of oxygen consumption relative to control animals as 0% change.

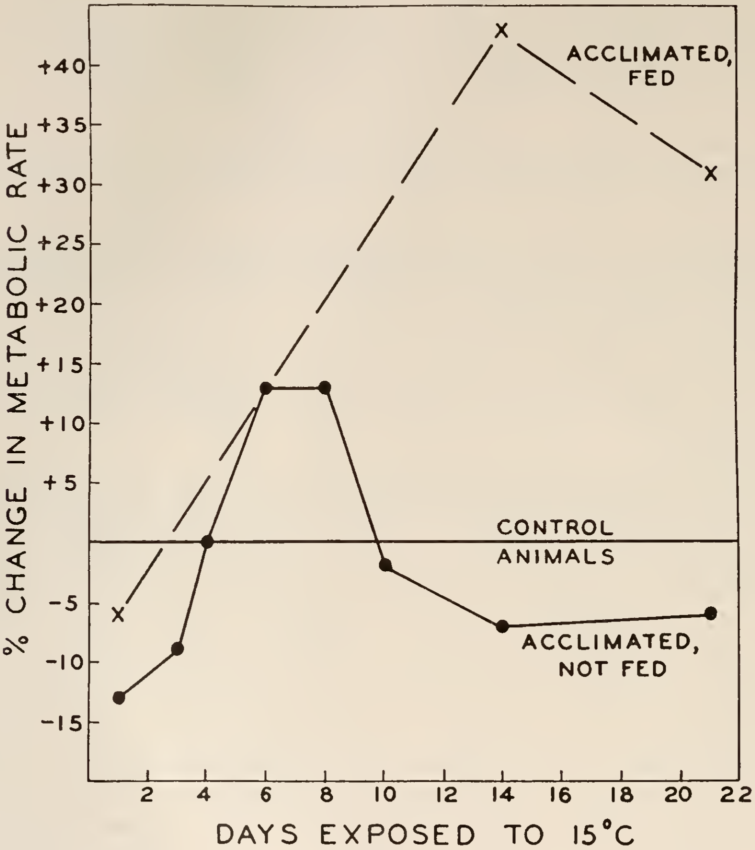

FIGURE 1. The influence of starvation during acclimation to 15° C. on the respiratory metabolism of *Uca pugnax* determined at 27° C. Percentage relationship of changes in rate of oxygen consumption relative to control animals as 0% change.

Their metabolic rate, which was initially depressed by cold, increased by day 6. The rate at day 10 was lower and fluctuated slightly during the remaining 11 days. A tendency to acclimate was apparent until days 6–8 and then was lost.

To determine the role of food in acclimation, this same experiment was repeated with one variation: food was presented to the 15 animals maintained at 15° C. on day 7 and day 11, while the rate of oxygen uptake was determined on days 1, 14, and 21. In a previous paper (Vernberg, 1959), it was shown that their metabolic rate dropped markedly after one day of starvation. This trend continued until day 3–5 when a leveling-off occurred with minor fluctuations observed until the end of the experiment on day 21. Thus in the present study, it was felt that the metabolic rate determined on day 14 did not reflect post-absorptive effects. Results, which are included in Figure 1, show that food had a pronounced influence on metabolism during temperature acclimation. The metabolic rate did not decline after day 6–8 as observed in the unfed animals, but continued high until the termination of the experiment on day 21.

Table I represents oxygen consumption data on animals which had been acclimated to 15° for various periods of time. The per cent change in metabolism is relative to values obtained for control animals. In this series of determinations, control animals are defined as warm-acclimated animals whose metabolic rate had been determined at various thermal levels. In all cases, these animals are starved for three to five days before determinations are made. For example, animals acclimated to 15° for 21 days consume oxygen 37% faster than warm-acclimated fiddler crabs when determined at 7° C. A significant difference in metabolic rate during cold acclimation was observed only at a few points. At 7° a marked increase was noted within 24 hours, but it was not until 21 days that the difference in the rate of oxygen consumption was significant at the 1% level. Although individuals exposed for 11 days or 21 days used oxygen at a faster rate than room-animals when determined at 17°, significant differences (at the 5% level) were observed only with 1 day animals. Results obtained at 27° were discussed above. Slight differences were observed at 33° and 39°.

TABLE I

The influence of acclimation to 15° C. for various periods of time on the respiratory metabolism of Uca pugnax determined at different temperatures. Percentage relationship of changes in rate of oxygen consumption relative to control animals as 0% change

Temperature ° C.	No. of days at 15°	% change
7	1	+21
7	21	+37*
17	1–2	+24**
17	11	+11
17	21	+16
27	1	−13
27	6	+13
27	14	+43*
33	1	− 8
33	9–11	+ 4
33	18–21	− 3
39	1	− 9
39	11	− 1

* Mean is significantly different from control at 1% level.
** Mean is significantly different from control at 5% level.

When determined at low temperatures (7° and 17°) the metabolic rate of cold-acclimated animals was always higher than control animals, but at elevated temperatures (33° and 39°) the reverse was observed (Fig. 2). At an intermediate temperature of 27° the response was intermediate; first the rate was depressed, as was the response at elevated temperatures, and then the rate increased with exposure to cold, much like the response at lower temperatures. The points on Figure 2 for cold-acclimated animals at 7°, 17° and 27° represented the maximum respiratory response to cold.

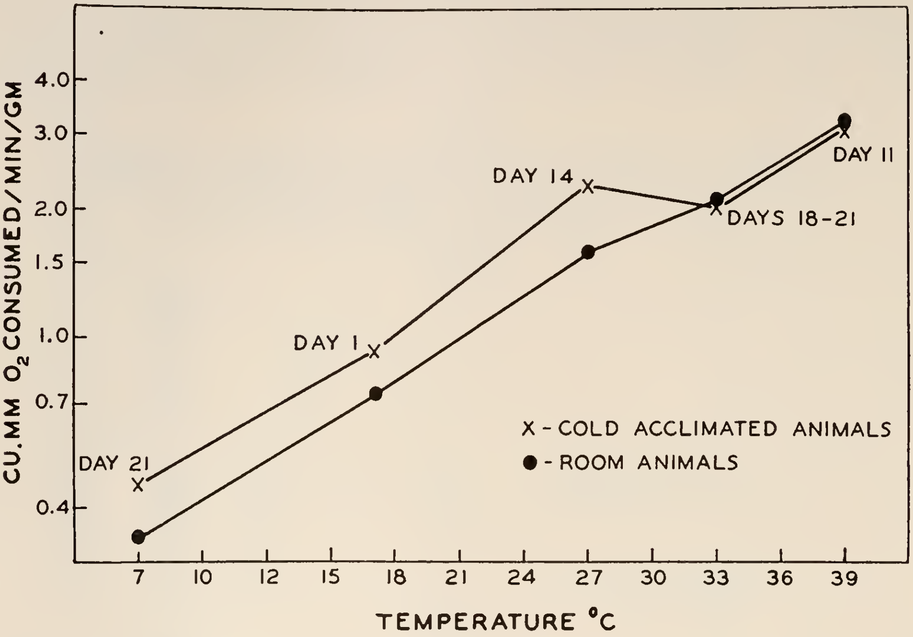

FIGURE 2. The oxygen consumption at various temperatures of room-animals and cold-acclimated *Uca pugnax* from Beaufort, North Carolina. Cold-acclimated animals had been kept at 15° C. for the periods of time indicated.

Uca rapax from Jamaica

The metabolic rate was determined at 28°, the animals were subjected to 15° for specific intervals of time, and then their metabolic rate determined again at 28°. At the beginning of this experiment 26 animals were used, but this number decreased with time due to death or discarding of animals which had lost appendages: three animals lost their large chela, two animals were dead after three days at 15°, five more had died by the fifth day, and three additional animals had died by day 7. No determinations were made after day 7 as the mortality level was too high. As a more critical method of analysis, the averages for the different days were computed only on the animals surviving throughout a particular time interval, *i.e.,* by day 5 only 18 animals survived, thus the averages cited in Table II reflect the data on only these 18 animals. Results of this experiment show that significant variation was observed only after one-day exposure to 15° and then only at the 3% level. It should be noted that in Table II the figures for the average metabolic index are not in terms of cu. mm. of O_2/min./gram as were the data on *U. pugnax*. These figures represent the average of the total number of manometer units change divided by the total elapsed time in minutes. For each successive daily determination an animal was placed in the same flask. Thus relative values could be used, and it was not necessary to multiply by the flask constant and divide by the weight of the animal to determine the absolute metabolic rate.

TABLE II

The influence of acclimation to 15° for various periods of time on the respiratory metabolism of Uca rapax from Jamaica when determined at 28°

Number of animals	Number of days at 15°	Average metabolic index	S.E.*
26	0	0.504	0.063
26	1	0.676**	0.048**
23	0	0.518	0.070
23	3	0.544	0.043
18	0	0.504	0.070
18	5	0.577	0.038
13	0	0.484	0.065
13	7	0.523	0.070

* S.E.—Standard Error.
** Means are significantly different at 3% level.

When determining the rate of oxygen consumption at 15° and then after exposure to 15° for one and five days, no significant fluctuation was observed. Similar rates of oxygen uptake determined at 12° were noted for 25 animals kept at room temperature and for 17 animals exposed to 15° for five days. Although temperature acclimation produced no apparent modification in metabolism when determined at low temperatures, marked changes were observed at higher temperatures. After five days at 15°, 19 cold-acclimated crabs consumed oxygen at a significantly higher rate than 26 animals maintained at room temperatures (at the 1% level) when determined at 36° C. These results are graphically represented in Figure 3.

Uca rapax from Alligator Harbor, Florida

Table III presents data on the metabolic response of *Uca rapax* from Alligator Harbor, Florida, after exposure to 15° for various periods of time. Unlike Jamaican forms, but similar to the more northern species, a significant drop (at the 1% level) in metabolic rate resulted after 24 hours at this reduced temperature. By days 3 and 8 no significant difference between non-acclimated and acclimated animals was noted. However, further exposure to cold resulted in a decreased rate which by days 12–14 was significant at the 5% level. Mortality was prac-

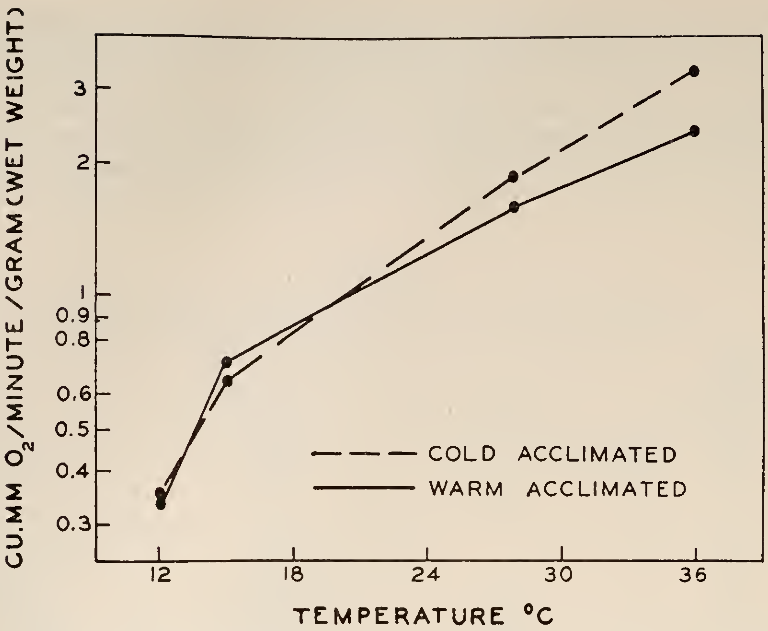

FIGURE 3. The oxygen consumption at various temperatures of warm- and cold-acclimated *Uca rapax* from Jamaica, The West Indies.

TABLE III

Metabolic response of Uca rapax from Alligator Harbor, Florida, after exposure to 15° C. for specific periods of time. Determinations made at various temperatures and results expressed as cu. mm. of O_2 *consumed/minute/gram of wet weight*

Temperature ° C.	No. days at 15°	Rate of oxygen consumption and standard error	No. of determinations	Significance of difference of means
35	0	2.901 ± 0.149	31	1% level
	11–14	2.026 ± 0.177	16	very significant
28	0	1.916 ± 0.087	58	3% level
28	1	1.695 ± 0.051	58	significant
28	0	1.856 —	36	not significant
28	3	1.666 —	36	
28	0	2.017 ± 0.102	26	not significant
28	8	1.802 ± 0.085	26	
28	0	2.438 ± 0.102	36	5% level
28	12–14	2.144 ± 0.108	36	significant
17	0	1.017 ± 0.066	30	5% level
17	29–53	1.217 ± 0.073	30	significant
7	0	0.352 ± 0.019	24	1% level
7	33–54	0.460 ± 0.011	16	very significant

tically negligible during this two-week period, which is in sharp distinction with the response of tropical forms.

Figure 4 represents the mean metabolic rate of cold-acclimated and room-animals determined at various temperatures. The means are significantly different for these two groups of animals at the four temperature points studied. At 7° and 35°, significance is at the 1% level, while at 17° and 27°–28° significance is at the 5% level. Determinations at 7° and 17° were made on animals maintained at 15° for 29–53 days. No apparent difference was observed in the response of 29 day or 53 day animals; this suggests that acclimation is complete within this time. At 27°–28° and 35°, animals had been kept for 11–14 days. Although greater metabolic differences might have been observed if animals had been maintained at 15° longer, it was felt that on the basis of work on *Uca pugnax* from North Carolina this period of time was sufficiently long to produce marked changes in metabolism.

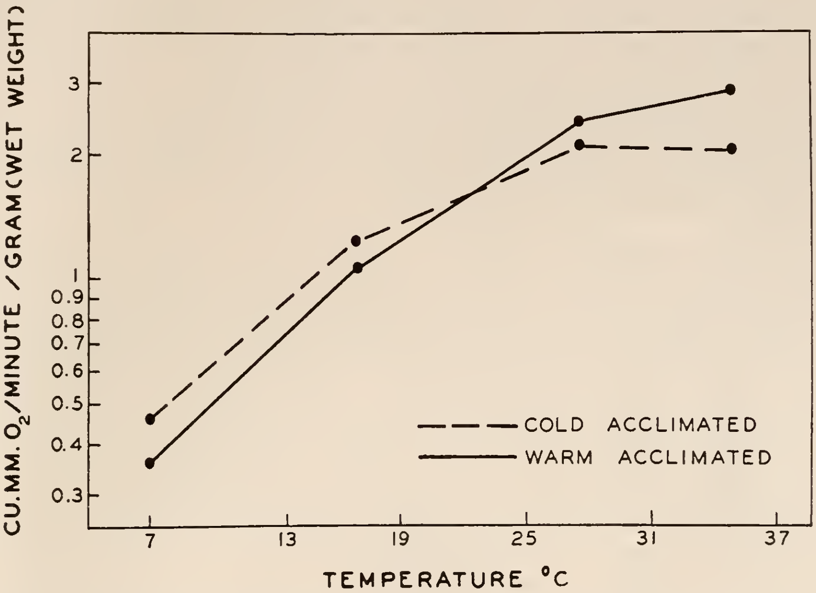

FIGURE 4. The oxygen consumption at various temperatures of warm- and cold-acclimated *Uca rapax* from Alligator Harbor, Florida.

DISCUSSION

Results of this study on the influence of thermal acclimation on respiratory metabolism furnish further evidence of pronounced physiological differences between temperate and tropical zone fiddler crabs. This work is in general agreement with the findings of other workers in this field.

Roberts (1957), working with population samples of *Pachygrapsus crassipes* collected from southern California to Oregon, found a close correlation between compensatory respiration levels and habitat temperature during the winter. Northern crabs consumed oxygen at a faster rate than southern forms when determined at an acclimation temperature higher than the field temperature at the time of collection. Although the experimental conditions in the present study are different from those of Roberts (1957), the same general tendency exists for individuals of the northern population to exhibit higher metabolic rates at low and intermediate temperatures than southern members of the same species. Roberts worked with a species inhabiting the temperate zone and thus subjected to marked seasonal temperature fluctuations, while the *Uca rapax,* used in the present study, extends from the tropics, where the temperature varies slightly during the year, to northern Florida with its seasonal thermal changes. Therefore, it is not surprising to note that the pattern of response of *U. rapax* from Florida is not like the tropical crabs, but more similar to that of *U. pugnax* from North Carolina, especially at temperatures below 17°. It would appear that with a selection pressure for temperature adaptability, a similar metabolic pattern has evolved in temperate zone forms. Although the response of these organisms might be similar, as measured by the oxygen consumption of the whole animal, the mechanisms governing the utilization of oxygen by the cells in these two species may be entirely different.

Uca pugnax appeared to require two weeks to be completely acclimated to low temperature (15°), which shows remarkable similarity to results of Roberts. When comparing these two papers it should be kept in mind that the absolute temperature referred to is not of paramount importance; the relative response of the organisms is the major consideration. In the study of *Pachygrapsus* the temperature (16°) used to base the degree of acclimation to warm or cold was close to the environmental mean for this species. However, in North Carolina the mean habitat temperature of *U. pugnax* during the summer is about 27°.

In both species, *U. pugnax* and *P. crassipes,* cold-acclimated forms consumed oxygen at a faster rate than warm-acclimated animals when determined over a wide temperature range. The only difference was that at elevated temperatures cold-acclimated *U. pugnax* consumed oxygen at the same rate as room-animals, whereas cold-acclimated *P. crassipes* continued to show greater respiratory activity than warm-acclimated forms. Although this difference in response is difficult to explain, it may be associated with the differences in environmental temperature extremes encountered by the two species. At Beaufort, North Carolina, the mean monthly water temperature varies from 5.5° in February to 28° in August (McDougall, 1943), while data presented by Roberts show yearly changes of a low of about 13° to a high of about 18° for Point Hueneme, California.

Working with *U. pugilator* from Massachusetts and Florida, Démeusy (1957) measured their respiratory rates at 1.4° and 15° after being maintained at 20° for various time intervals. The northern forms showed higher rates throughout a seven-week period when determined at 1.4°. Although no statistically significant difference was noted between the respiratory activity of the two groups of fiddler crabs at 15°, it should be noted that determinations were not made at this temperature over an extended period of time. In the present study, northern forms of *U. rapax* consumed oxygen at a higher rate than southern forms, especially at lower temperatures, which is similar to the findings of both Démeusy and Roberts.

In contrast to the results of Roberts, Démeusy found no decrease in metabolic rate of crabs from different localities when kept at a common temperature. She felt that regular feeding of the animals might be responsible for this steady rate of metabolism. Results of the present paper demonstrate the following: (1) feeding of *U. pugnax* during acclimation to 15° resulted in the maintenance of a steady rate of oxygen consumption after two weeks; and (2) the temperate zone forms of *U. rapax* showed a gradual decline in metabolic rate during acclimation to 15°, as shown by Roberts, while the tropical animals showed a tendency to have higher rates.

The respiratory activity at 1.4° of *U. pugilator* from Florida decreased slightly for the first seven days of acclimation to 10° when compared with crabs kept at room temperature. This decrease was followed by a slight increase after one week. Although *U. rapax* from Florida and *U. pugnax* were subjected to slightly different experimental conditions, cold-acclimated animals of both species consumed oxygen at significantly higher levels than room-animals when determined at 7°. This response is similar to findings of Roberts on *Pachygrapsus*. However, the respiratory pattern of the tropical form of *U. rapax* did not change during acclimation to 15° except at elevated temperatures.

Recently Teal (1959) reported that on the basis of studies on the crabs found in one area, a Georgia salt marsh, *U. pugnax* had a better developed mechanism for thermal acclimation than *U. pugilator*. This might help explain some apparent differences between the results of Démeusy and those of the present paper.

Prosser (1958) has described various patterns of temperature acclimation based on measuring reaction rates over a range extending beyond the acclimation temperature. Using his terminology, it is possible to characterize the pattern of response of the tropical and temperate zone fiddler crabs used in the present study.

Tropical forms of *U. rapax* appear to best fit pattern I (no adaptation) except between 28° and 36° where the Q_{10} of cold-acclimated animals is increased significantly over that of warm-acclimated organisms, suggesting pattern IIIB. Similar results have been reported by Scholander *et al.* (1953), when comparing some tropical and arctic terrestrial insects. The metabolic response of the temperate zone form of *U. rapax* appears to resemble pattern IIIA, which indicates that the Q_{10} of cold-acclimated animals is less than the Q_{10} of warm-acclimated forms. Démeusy (1957) found this response in *U. pugilator*. *U. pugnax* from North Carolina seems to fit pattern IVA best, which seems to be the most common method of temperature acclimation, according to Prosser. Rao (1953) observed this pattern in the rate of pumping water by *Mytilus* from different latitudes.

On the basis of this method of comparing patterns of metabolic response during temperature acclimation, certain generalizations might be made. Both populations of *U. rapax* demonstrated a rotation of rate function curves, whereas in *U. pugnax* the type of change is a translation of curves. This might suggest that different biochemical mechanisms are involved in these two species.

The results of Tashian (1956) on oxygen consumption of *Uca* from different latitudes indicate that different patterns may be found when making intra- and inter-specific comparisons of animals from different geographic locations. This was noted also in the present study. Intraspecific comparisons of *U. pugnax* from New York and North Carolina and *U. rapax* from Florida and Trinidad represent pattern IIA. An inter-specific comparison of *U. pugnax* from New York and *U. rapax* from Trinidad and Florida suggests pattern IIIA, while comparing *U. pugnax* from North Carolina and *U. rapax* from Florida seems to indicate pattern IVB. It should be noted that this work was done on animals with similar recent thermal histories and determinations were made at two temperatures. Vernberg (1959) measured the rate of oxygen consumption over a wide temperature range of 7 species of temperate and tropical zone fiddler crabs. He noted that systematic differences correlated with latitude were found only at lower temperatures. The findings of the present paper, which involved acclimation studies, suggest that with the evolution and distribution of animals to new environmental complexes a new pattern of acclimation has resulted.

SUMMARY

1. Studies on the metabolic response of tropical and temperate zone fiddler crabs (Genus *Uca*), after various periods of thermal acclimation, were done to assess the importance of this factor in the evolution and climatic adaptation of these forms.

2. *U. pugnax,* a temperate zone species, showed a significantly higher metabolic rate after acclimation to 15° than warm-acclimated forms when determined at 27°, 17° and 7°, but not at 33° and 39°. When animals were starved during the period of acclimation, the tendency to acclimate was apparent by days 6–8 and then was lost. Feeding maintained the pattern of acclimation for at least 21 days.

3. *U. rapax* from Jamaica, The West Indies, did not show any shift in metabolic response during acclimation to 15°, except when determined at 36°, where the rate of cold-acclimated forms was higher than warm-acclimated animals.

4. Population samples of *U. rapax* from Florida responded metabolically like temperate zone animals at low temperatures and the tropical animals at high temperatures.

5. Results of this study demonstrated that during the evolution and distribution of fiddler crabs different patterns of acclimation have resulted.

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RESPIRATION AND ANAEROBIC SURVIVAL IN SOME SEA WEED-INHABITING INVERTEBRATES[1, 2]

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L. C. Beadle in a paper on the respiration in the African swampworm *Alma emini* (Beadle, 1957) showed that the waterlogged mats of *Cyperus papyrus* are devoid of oxygen most of the time and that all animals living in them have to be adapted to this situation one way or the other. The case of the swamps is certainly an extreme one, but there can be no doubt that wherever plants grow in water or are easily flooded, anaerobic conditions may prevail occasionally or quite regularly.

The large sea weeds growing along the open coasts seem to represent an exception to such a statement and might be regarded as the prototype of a well-aerated habitat. This is certainly true at high tide. At low tide, however, these sea weeds collapse, forming thick clumps within and under which water is trapped. In the night or on cloudy days respiration of the algae may exceed photosynthesis and the water, cut off from atmospheric circulation, will gradually lose its oxygen to the plants.

This, then poses an ecological problem which can be stated as follows: Does the oxygen content of the water trapped by emersed sea weeds ever fall to zero and if so, how do the animals living in the sea weeds cope with this situation?

MATERIAL AND METHODS

The work was carried out at the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts. The water samples were taken from under clumps of emersed brown algae (*Fucus, Ascophyllum*) on July 13 and 14, 1959, by means of 5-cc. syringes which were stoppered with glass plugs, and analyzed in the laboratory with the gasometric water analyzer of Scholander (Scholander *et al.*, 1955). The sampling site was a flat piece of gravelly and rocky beach (Woods Hole Yacht Club) on which brown algae grew quite luxuriantly. All the samples came from the upper and the middle zone of the exposed algal belt. The tidal range at Woods Hole is 1.8–2.2 ft. The temperature within a clump of sea weeds measured 20° C. at one occasion.

Various animals living in the large brown sea weeds around the Woods Hole Oceanographic Institution were collected at high tide and their respiration and anaerobic survival studied. The respiration of nematodes and mites was measured by the Cartesian diver method as described by Holter and Linderstrøm-Lang (1943) and others. The divers used had a volume from 6.5 to 37.4 μl. and volume changes could be read to 0.001–0.006 μl. O_2/hr. The respiration of the

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[2] Contribution no. 1061 from the Woods Hole Oceanographic Institution.

amphipods was measured by means of the volumetric micro- and macro-respirometer of Scholander (Scholander *et al.*, 1952). Respirometer runs were made at 10° C. and in the following diagram the respiration values are corrected to 20° C., the temperature at which the diver was run, by assuming a Q_{10} of 2. This value seems to be a reasonable—perhaps even too low—guess in the light of the data on the amphipod *Talorchestia* by Edwards and Irving (1943) as re-plotted by Rao and Bullock (1954).

Anaerobic survival was studied by putting the animals into a small amount of water in a Thunberg tube which then was evacuated and flushed with tank-nitrogen. Constant experimental conditions were maintained by putting a constant amount of water into the Thunberg tubes and by flushing each tube exactly 100 times. The evacuation of the Thunberg tubes was carried out at room temperature. Subsequently, the tubes were kept either in the refrigerator at approximately 2° C., or at 15° C., or in the laboratory which had a fairly constant temperature of 25° C. The behavior of the animals was studied either by observing them in the closed tube, or after a certain time had elapsed, by emptying them into a dish with normal sea water. The recovery time of nematodes and mites was defined as the time interval within which 75% of the animals used for one experiment had resumed movement.

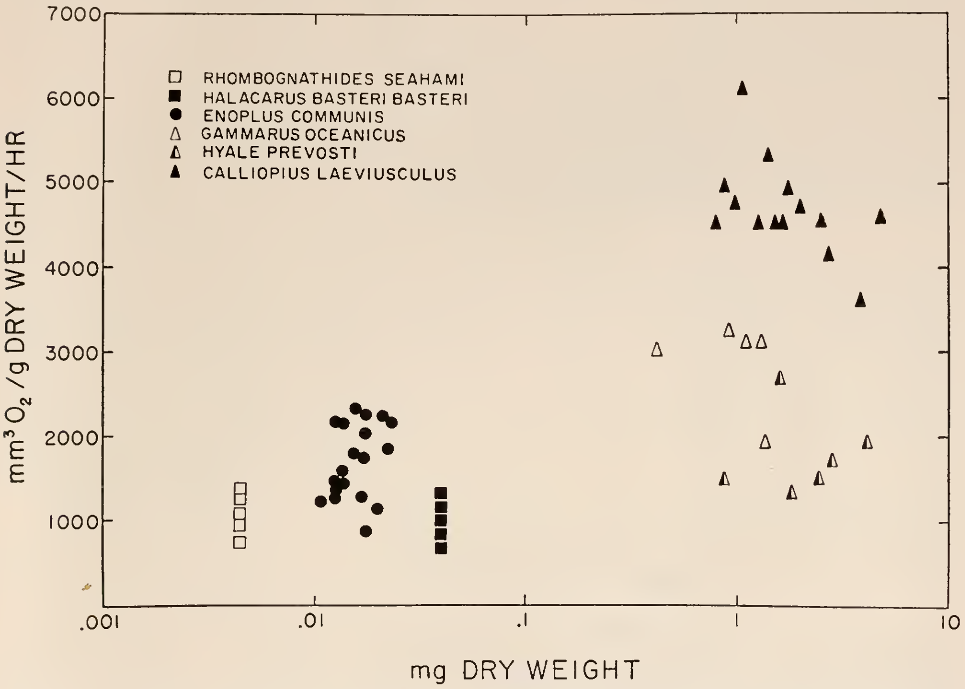

FIGURE 1. Oxygen consumption per gram dry weight of the six species of animals investigated. Each convention represents one experiment.

The dry weight of the nematodes was established by measuring volume and specific gravity as described elsewhere (Wieser, 1960). The dry/wet weight ratio

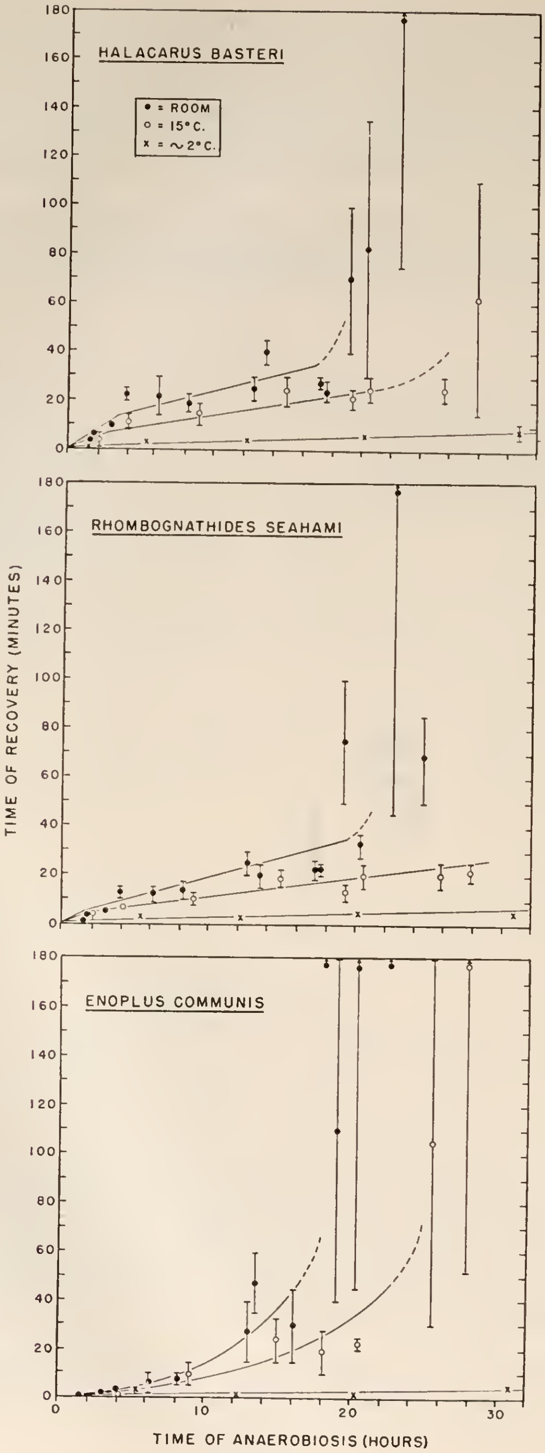
HALACARUS BASTERI
● = ROOM
○ = 15° C.
x = ~2° C.
RHOMBOGNATHIDES SEAHAMI
ENOPLUS COMMUNIS
TIME OF RECOVERY (MINUTES)
0
20
40
60
80
100
120
140
160
180
0
10
20
30
TIME OF ANAEROBIOSIS (HOURS)

FIGURE 2.

was assumed to be 25% in all specimens. The mites were dried at 100° C. and weighed in batches on a "Misco" quartz helix (by Dr. R. Conover, W.H.O.I.). The dried amphipods were weighed individually on a torsion balance.

RESULTS

The water analyses gave the following results:

Sample no.:	1	2	3	4	5	6
Time:	10 P.M.		10 A.M.			
Ml. O_2/1 liter H_2O:	0.25	0.30	0.0	0.05	0.96	1.65

The night samples were taken exactly at low tide, the day samples 30–40 minutes after low tide on a cloudy day (illumination 1700 foot-candles). The oxygen content of saturated sea water at 20° C. and the local salinity (approximately 31‰) is 5.5 ml. O_2/1 liter H_2O (Sverdrup *et al.*, 1946).

For the measurement of respiration and anaerobic survival two groups of species were chosen; one, consisting of the nematode *Enoplus communis* and the sea mites (Halacaridae) *Rhombognathides seahami* and *Halacarus basteri basteri,* represented very small animals of low motility, unable to swim and without special respiratory organs. The other group consisted of three amphipod species, *Calliopius laeviusculus, Gammarus oceanicus* (identified by Dr. H. Werntz, Harvard University) and *Hyale prevosti,* representing medium-sized, very agile animals with good swimming capabilities and equipped with gills.

The respiratory rates per gram dry weight of the six species investigated are summarized in Figure 1. It is evident that if the size difference is taken into account (Zeuthen, 1947), the amphipods have a much higher metabolic rate than the other three species.

The two groups of species could also be distinguished in their reaction to anaerobic stress. Nematodes and mites, after the oxygen had been removed from the Thunberg tube, became paralyzed. From this state they recovered after they were put back into oxygenated water, provided that the time of anaerobic stress had not lasted too long. The recovery time as shown in Figure 2 is a function of the time of anaerobiosis and of the temperature at which the latter was spent. The three amphipods, however, remained active under anaerobic stress for a certain time and then died (Fig. 3). One could, of course, say that they, too, became paralyzed after a period of activity, but that the paralyzed state which preceded death was of extremely short duration. If an animal after readmission to normal conditions did not recover right away, it did not recover at all.

FIGURE 2. Relationship between time of anaerobiosis and time of recovery in the two halacarids (upper two diagrams) and the nematode. Each convention represents one experiment on a batch of animals. Range of variability in recovery time indicates beginning of movements in the first and in 75% of the animals in one batch. Recovery periods exceeding 180 minutes are characterized by an arrow and by a circle close to the upper abscissa of the diagram.

The survival period of one amphipod, *Calliopius laeviusculus,* was extremely short, certainly not more than 5 minutes at a temperature of 25° C. The other two species, *Gammarus oceanicus* and *Hyale prevosti,* did considerably better, the survival period ranging from about half an hour of anaerobic stress to about three hours. There was no clear-cut relationship between survival time and the size of the animals.

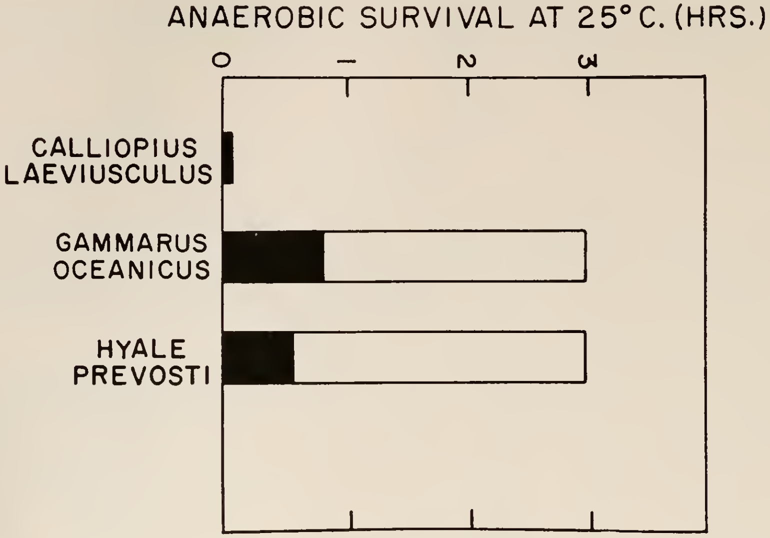

FIGURE 3. Survival under anaerobic conditions of the three species of amphipods studied. The black part of each column indicates the time of anaerobiosis *all* individuals survived, the white part indicates ranges of variability of survival time.

DISCUSSION

The water analyses show plainly that animals living in the water trapped by large sea weeds at low tide may be subject to oxygen-free or almost oxygen-free conditions. Under extreme circumstances (very large clumps of algae, no light, high temperature) these conditions probably set in soon after the receding tide has uncovered the plants and may last for several hours. A drop in the oxygen content to 30% saturation value was found by Revelle and Emery (1958) in inter-tidal basins with algal growth at the bottom.

Animals living in sea weeds react in different ways to the movements of the tides. As shown previously (Wieser, 1952), some animals remain in the sea weeds all the time while others (in Plymouth, England, *e.g.* copepods and the amphipod *Stenothoe monoculoides*) leave their habitat with the receding tide and return to it with the rising tide. To the former group, obviously, belong slow-moving, non-swimming animals like the nematode and the mites studied in this paper. These species are adjusted to their environment by being able to withstand anaerobic conditions at 25° C. for at least 12 hours which, in an area with diurnal tides, is the theoretical maximum their habitat could remain emersed.

That paralysis during anaerobiosis is due to the accumulation of toxic metabolites within the body has been pointed out by von Brand (1946). Since the accumulation of metabolic substances is a temperature-dependent process, the close dependence of the rate of recovery from paralysis on the temperature at which the anaerobiotic period was spent (Fig. 2) could have been anticipated (see also Miller, 1957). Furthermore, the recovery time from anaerobiosis is a characteristic of each species. In the two halacarids, a linear relationship exists between the time of anaerobiosis and the time of recovery, up to the point at which the effects of asphyxiation become lethal and the curve expressing the relationship turns sharply upwards (Fig. 2). The slope of the linear portion of the curve is steep in the beginning and flattens out somewhat after 2–4 hours' application of anaerobic stress at room temperature. The points of inflection of the triphasic curve and the absolute values of recovery time, but not the slopes of the curves, are different in the two species of Halacaridae. However, the triphasic curve is well defined only for the experiments at room temperature. The 10-times larger species of *Halacarus* is more susceptible to the effects of asphyxiation than the species of *Rhombognathides.*

The relationship between time of anaerobiosis and time of recovery in the nematode *Enoplus communis* is an exponential one.

Kalmus (1942), in similar experiments, reported the recovery time to be different in different species and even mutants of *Drosophila.* In this genus, as in the halacarids over a certain period, a linear relationship between time of anaerobiosis and time of recovery was observed.

The second group of species, which consists of animals obviously able to leave and to repopulate the sea weeds at will, does not have to be adjusted for survival of anaerobic periods. The efficiency of receptor and locomotor organs of amphipods and related forms can always be considered as being sufficient to keep the animals in oxygenated water all the time. Accordingly *C. laeviusculus* has almost no resistance to the reduction of oxygen in its environment. The data in Figure 3 show that even five minutes of anaerobic conditions bring about the death of all members of this species, but the other two species of amphipods show some resistance. A related species of *Gammarus, G. duebeni,* is even known to occur on European coasts in rock pools and stagnant waters smelling of H_2S (Kinne, 1959).

The data of Figure 1 show that the least resistant amphipod, *C. laeviusculus,* has twice the oxygen consumption of the other two amphipod species. This might reflect the fact that the former species also leads a pelagic life, particularly in winter (Kunkel, 1918).

In a very general way it can be concluded that the nematode and the two halacarids in an anaerobic environment produce and deposit metabolic breakdown products which are toxic and cause paralysis. Up to a certain time, however, the tissues are not irreversibly injured by these breakdown products. The failure of *Calliopius laeviusculus* to endure even very short periods of de-oxygenation might be due either to its inability to gain sufficient energy under anaerobic conditions, or to the irreversibly harmful effects of even very small amounts of the breakdown products of anaerobic metabolism. The behavior of the remaining two amphipod species could be explained by assuming that either their energy or their excretory mechanisms function imperfectly under anaerobic conditions.

Summary

1. The water trapped by large brown sea weeds at low tide may become oxygen-free in the night or on cloudy days.

2. Slow-moving, non-swimming animals living in the sea weeds all the time (like nematodes and mites) are paralyzed by the removal of oxygen from the water, but they recover from this state if the period of anaerobic stress has lasted less than approximately 16 hours at 25° C. The relationship between time of anaerobiosis and time of recovery is temperature-dependent and a characteristic of each of the three species investigated (*Enoplus communis, Rhombognathides seahami, Halacarus basteri basteri*).

3. Agile animals, capable of leaving and repopulating the sea weeds with the tides, show a different reaction pattern. One of the amphipods investigated (*Calliopius laeviusculus*) did not survive even a few minutes of anaerobiosis, while the other two species (*Gammarus oceanicus, Hyale prevosti*) survived from about half an hour to three hours of anaerobiosis. The former species occasionally leads a pelagic life and has twice the respiratory rate of the latter two species.

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ROLE OF LIGHT IN THE PROGRESSIVE PHASE OF THE PHOTOPERIODIC RESPONSES OF MIGRATORY BIRDS[1]

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Two conspicuous changes in physiological state precede the initiation of northward migration in late March in the slate-colored junco (*Junco hyemalis*)—gonadal recrudescence and fat deposition. The occurrence of these changes is regulated by day-length and in two separate phases. The first phase, called the preparatory phase, occurs in the fall and requires short days for its completion. The second phase, called the progressive phase, occurs in late fall and winter, and the rate at which it proceeds is a function of the daily photoperiod and is extremely rapid under long days. The summation of different degrees of physiological response to the daily light-dark cycles has been postulated to explain the events in these phases. (See Wolfson, 1959a, 1959b for review and references.)

The observations that interruption of the long night of a short day with a brief period of light during the progressive phase results in a rate of response comparable to that of a long day (Kirkpatrick and Leopold, 1952; Jenner and Engels, 1952) and that administration of the same total photoperiod in smaller doses induces a more rapid response (Wolfson, 1953), raised the question of the roles of light and darkness. The experiments reported here are concerned with the progressive phase and are part of an extensive series which was designed to determine the roles of light and darkness in both the progressive and preparatory phases. A brief summary of the results of all previous experiments and a preliminary report of one aspect of the present experiments have been reported (Wolfson 1959a, 1959b, 1959c).

The roles of light and darkness were explored in the present study by combining effective daily photoperiods with inhibitory dark periods and testing their effects in the winter and spring.

MATERIALS AND METHODS

The species used was the slate-colored junco. Its geographic distribution and migration and the sequence of events in its annual cycle have already been described (Wolfson, 1952a). The birds were trapped during the period of fall and spring migration and were held in captivity under natural day-lengths (including civil twilight) until the experiment began. Artificial illumination only was used in all experimental rooms and was provided by 40-watt white fluorescent bulbs. Intensity varied from approximately 15 to 60 foot candles, depending on the dis-

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tance of the cages from the light source. Temperature was not controlled and the rooms were unheated. In the winter experiment, the temperature ranged from 67°–79° F.; in the spring experiment it ranged from 60°–80° F. and was usually in the low 70's. Conditions of captivity, feeding, and care were similar to those already reported. Observations were made on fat deposition, body weight, and reproductive activity using methods described previously (Wolfson 1952a, 1954).

EXPERIMENTS AND RESULTS

Experiment 1. In this experiment a stimulatory photoperiod, 12 hours, was combined with an "inhibitory" dark period, 16 hours, to give a 28-hour cycle. The experiment began on December 11 with 19 juncos and was continued to May 27, well beyond the normal time of response to experimental treatment, in order to determine the duration of the response, which is diagnostic for a 12-hour photoperiod. By the starting date, the birds had completed the preparatory phase.

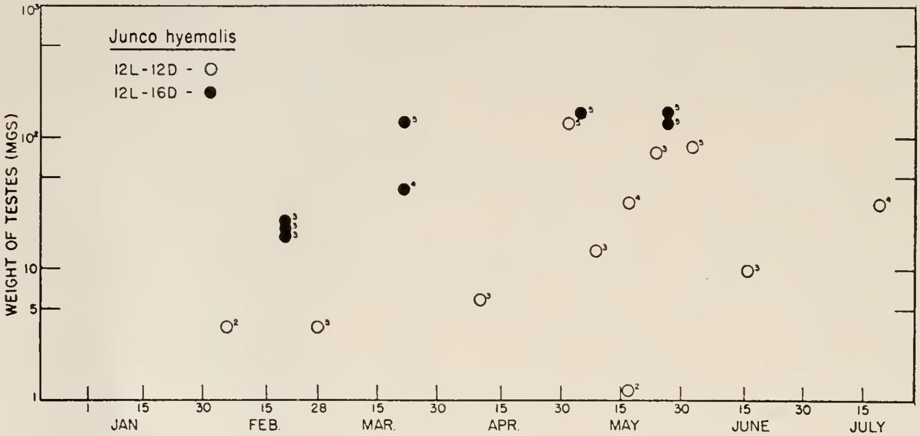

FIGURE 1. Testicular response in the light-dark cycles indicated. Weight of testes on logarithmic scale. Numbers adjacent to points indicate stage of spermatogenesis; stage 1 is minimum and 5 is maximum.

Sixteen (11 ♂♂, 5 ♀♀) of the 19 birds showed a gonadal response. Figure 1 shows the rate of response for the responsive males and permits comparison of this rate with that of juncos treated with 12L–12D cycles beginning December 4 (Winn, 1950). The rate of response was clearly greater in the present experiment. The duration of response, however, was similar and was longer than that in birds treated with approximately 16-hour photoperiods, in which testicular activity ends by March 15 (Wolfson 1959b). The seminal glomera (vesicles) were not well developed, not even in the three birds autopsied in May which showed complete spermatogenesis (Stage 5). Histological study revealed normal spermatogenesis except perhaps in two males (autopsied on May 27) in which the germinal epithelium was extremely low and the lumens of the tubules extremely large. The mean ovarian weight for four responsive females autopsied in April and May was 26.3 (21.0–36.2) grams.

Seventeen of the 19 birds also showed a typical vernal fat response and concomitant increase in body weight. Figure 2 shows the mean body weights for the females and two groups of males, one of which responded sooner. The mean increase in weight (and range) from initial weight to maximum response weight (when birds were in the heavy fat class) was 5.2 (2.7–6.6) grams for 12 males and 5.4 (4.7–6.1) grams for 5 females. For the three groups graphed in Figure 2, the mean increases for each were, respectively, 5.6, 5.6, and 5.4 grams, and each represented a 31 per cent increase from the initial weight. Using the median date between successive weighings when the birds first showed moderate or heavy fat deposits, it took from 15–83 days for the fat response; the mean for the central group of seven males was 54 days; for a similar group of four females it was 48 days. Comparable figures from the previous 12L–12D experiments (two separate experiments, and without separation of data for each sex) were 61 days and 79 days, and in these experiments only two males in a group of 23 responded in less than 55 days. The mean increase in weight for 12 birds in one experiment was 5.6 grams, which is almost identical with that in the present experiment.

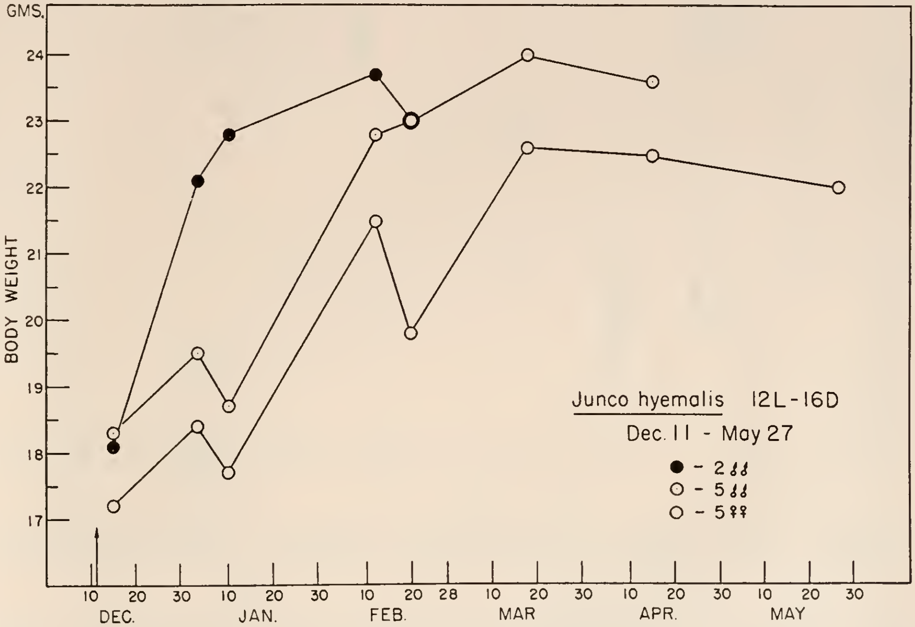

FIGURE 2. Changes in body weight in the 12L–16D group from December 11 to May 27. Each point is the mean weight for the three groups shown in the legend.

Experiment 2. From the results of experiment 1 it seemed likely that the photoperiod determined the response and that the dark period was not inhibitory. But a possible weakness in that experiment was the ratio of light to darkness, which was close to one, and hence simulated a 12L–12D cycle. In an 8L–16D schedule, when the dark period appeared to be inhibitory, the ratio of dark to light was 2:1. To test further, therefore, the following schedules were selected, using longer dark periods: 12L–20D; 16L–16D; 16L–22D; 16L–32D. The 16-hour photoperiod was selected because it is more stimulating than a 12-hour photoperiod; the 16L–32D cycle was chosen to obtain the same ratio of dark to light as in the nonstimulatory 8L–16D cycle. The lights were controlled automatically by time switches prepared specially for this study by the Aemco Corporation (Mankato, Minnesota).[2]

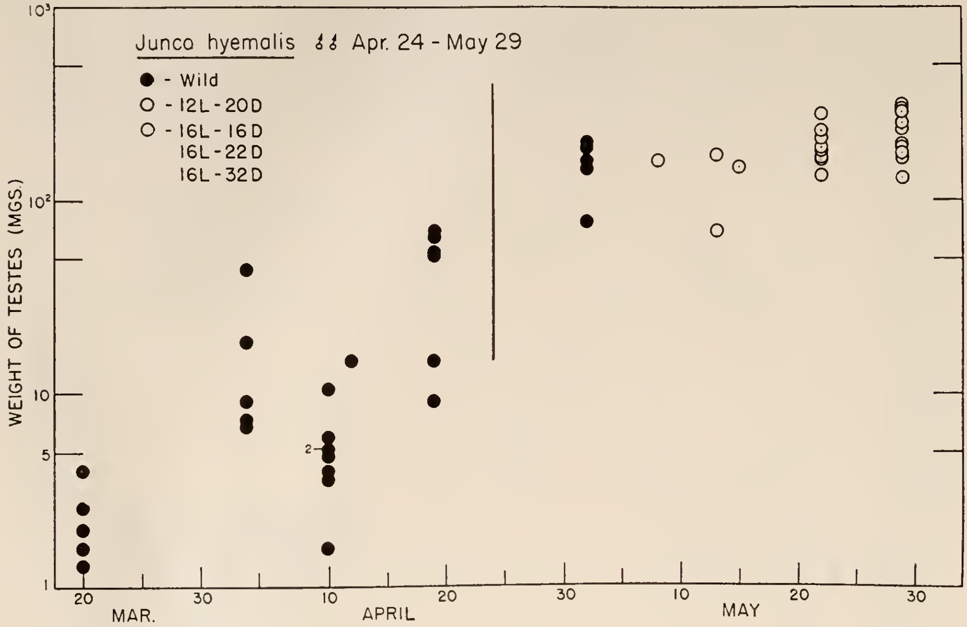

FIGURE 3. Weights of testes in wild and experimental birds in spring. Weights of testes on logarithmic scale. Vertical line indicates time of initiation of experimental light-dark cycles indicated in the legend.

This experiment was performed in the spring when gonadal growth and fat deposition were already underway, but continuation and maintenance of these responses are also regulated by photoperiod. For example, treatment with long daily dark periods, as in a 9L–15D cycle, beginning in late April, results in gonadal regression and loss of fat deposits within a few weeks; in cycles of 12L–12D, 20L–4D, and under natural day-lengths the responses continue, with a slower rate and a protracted response under 12-hour photoperiods.

The experiment began on April 24 and ended May 29; 68 juncos were used. The number and sex of the birds in each group were as follows: 12L–20D—11 ♂♂, 10 ♀♀; 16L–16D—3 ♂♂, 8 ♀♀; 16L–22D—6 ♂♂, 11 ♀♀; 16L–32D—2 ♂♂, 12 ♀♀.

Gonadal data were obtained for 22 ♂♂ and 41 ♀♀. The response of the males is shown in Figure 3, that of the females in Figure 4. Also shown are gonadal data from wild birds and birds held under natural day-lengths to indicate the normal rate of growth and the condition of the gonads when the experiment began. It is clear from Figure 3 that all of the males maintained their reproductive activity. Histological study showed that spermatogenesis was normal except perhaps in a few cases where the lumens of the tubules were larger than normal because of the decreased depth of the germinal epithelium. There was a slight retardation in spermatogenesis in three birds, since they attained only stage 4 by the end of May. One occurred in each of the groups except the 16L–16D. The seminal glomera were at maximal or submaximal size by May 29, except in a few cases. The testes of birds which received only 12 hours of light daily were as well developed as those in birds which received 16 hours of light.

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[2] In a later study it was discovered that the light period in the 16L–32D cycle was 20–25 minutes shorter and the dark period correspondingly longer. This difference does not affect the design or results of the experiments, and for convenience the cycle is still referred to as 16L–32D.

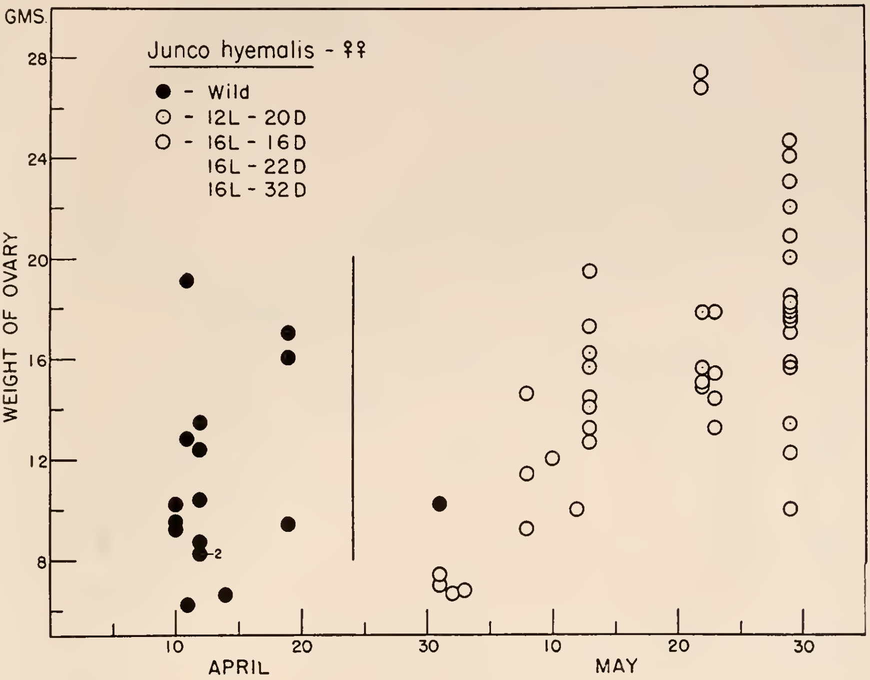

FIGURE 4. Weights of ovaries in wild and experimental birds in spring. Vertical line indicates time of initiation of experimental light-dark cycles.

The response of the females (Fig. 4) was more variable, but the results clearly indicate continuing growth of the ovary and maintenance of activity in all birds. The size of the largest follicles ranged from 1–2 millimeters by late May; this is the normal maximum size in captive birds. Inactive ovaries normally weigh less than 10 milligrams. The mean weight (and range) for four inactive ovaries obtained in the first 10 days of the experiment was 7.0 (6.6–7.4) milligrams.

Of the 68 birds in the experiment, 64 lost weight and reached below-normal weights which ranged from 12–15 grams. Only four birds failed to show abnormal losses in weight, and three of these were in the 16L–16D group. Representative data for individual birds are presented in Figure 5, which shows the weight changes in the males in the 12L–20D group. The individual curves show the uniformity of response. The weight loss, to about 18 grams, was due mostly to loss of fat from subcutaneous and intraperitoneal depots. The mean loss was about 6 grams and the percentage lost (from the initial mean weight) was about 31 per cent. Adequate numbers of females occurred in all groups to permit calculation of the mean weight curves which are given in Figure 6. Marked losses occurred in all groups, reaching abnormally low levels especially in the 12L–20D and 16L–22D groups. The weight loss of 32 per cent in 12L–20D females was almost identical to that in the males.

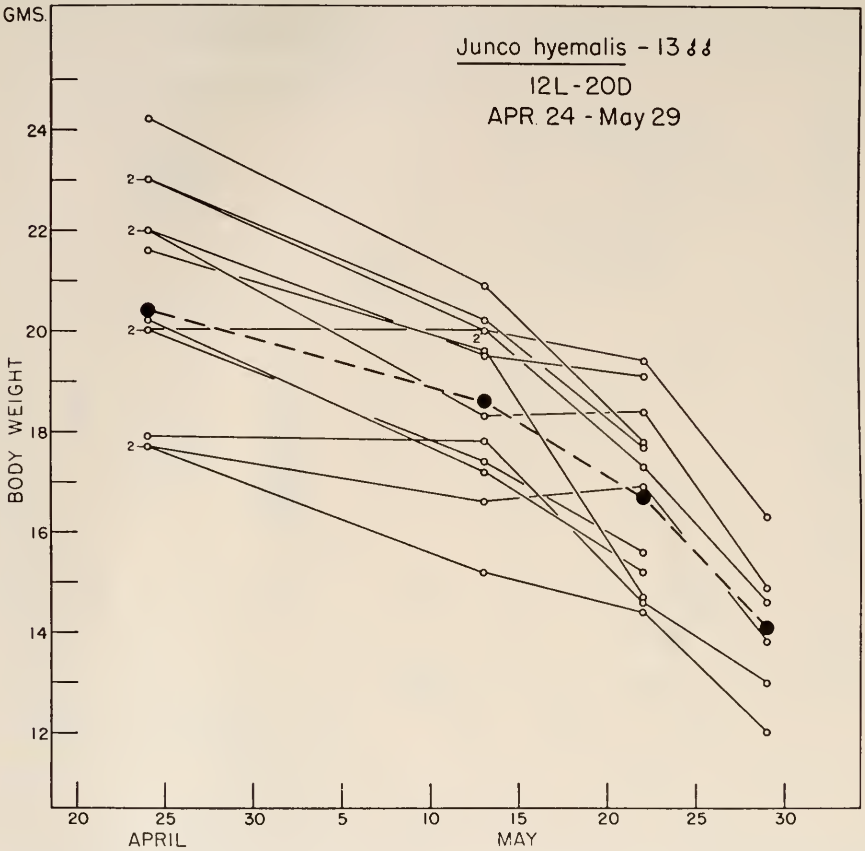

Figure 5. Changes in body weight in individual male juncos treated with 12L–20D cycles in spring. Mean for the group shown by large points and dashed line.

It is evident that the gonadal response continued despite excessive losses in weight. For 12 birds that showed the greatest gonadal weights on May 22 and May 29, the mean weight loss was 5–6 grams except in the 16L–16D group where it was 2–3 grams. No. 464 in the 16L–22D group had the largest testes of all the birds on May 29 (312.6 milligrams); its body weight was 13.4 grams, which was 6.6 grams less than its initial weight.

Discussion and Conclusions

From the results of their studies Kirkpatrick and Leopold (1952) and Jenner and Engels (1952) assigned an inhibitory role to the long dark period of short winter days. On the basis of the experiments reported here it seems unlikely that the dark period is inhibitory. The responses simulated those obtained with similar photoperiods rather than similar dark periods. This was brought out particularly by the 12L–16D schedule in the first experiment. Not only did the birds respond, but the duration of the response was longer than normal and characteristic of 12-hour photoperiods (Wolfson, 1952b, 1959b). Also, the annual molt was not induced and this also is characteristic of exposure to 12-hour photoperiods. By contrast, birds treated with approximately 16-hour or longer photoperiods beginning in December showed a shorter duration of response and were usually molting by April 1.

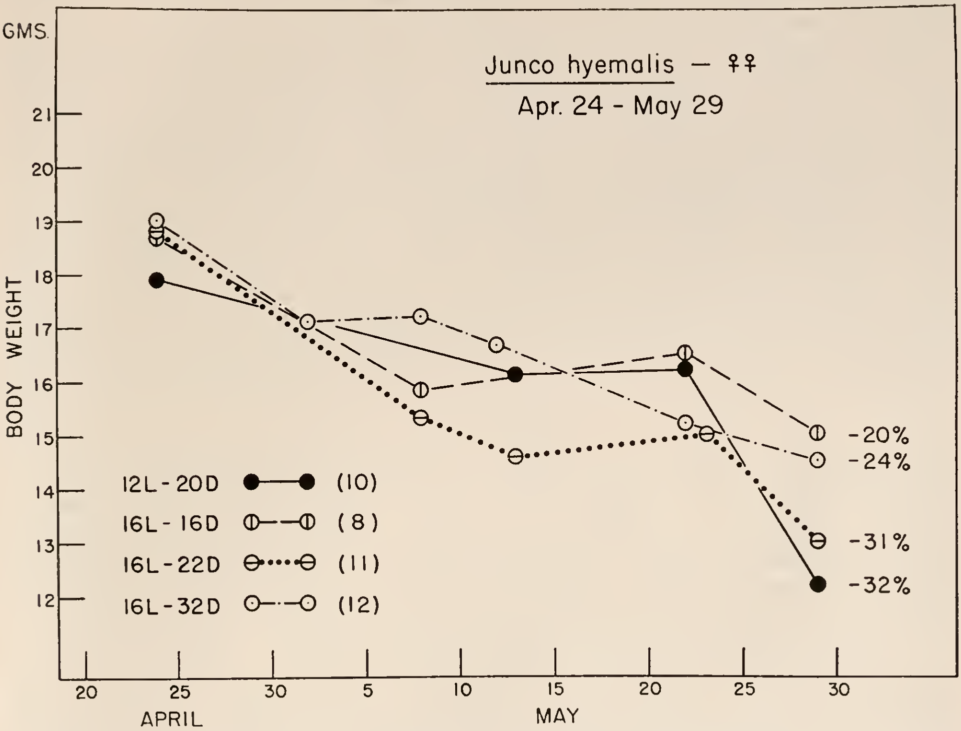

Figure 6. Changes in mean body weight in females in the light-dark cycles indicated. Number of birds in each group is given in parentheses in the legend. Percentage lost is based on mean initial weight.

In the spring experiments, long dark periods also failed to inhibit gonadal activity. It is clear that an effective photoperiod, for example, 12 or 16 hours long, can maintain gonadal activity despite long intervening dark periods, even when the dark period is twice as long as the light period, as in the 16L–32D schedule. The ability of the birds to maintain gonadal activity under this schedule raised the question of whether such a schedule could induce the gonadal response in winter. This was tested recently, and not only was a gonadal response obtained but also a fat response (Wolfson and Winchester, unpublished data). The occurrence of the fat response was completely unexpected, since the birds lost weight in the present experiment. Again, a long dark period failed to be inhibitory when coupled with an effective photoperiod.

When the rate of gonadal response in the 12L–12D and 12L–16D groups (Fig. 1) was compared, it was surprising to find a faster rate when the dark period was longer. Analysis of the time of the fat response in the two groups also showed a clear tendency for a faster response in the 12L–16D group. It is interesting that although the rate of the gonadal response in the 12L–16D group was similar to that under 16-hour photoperiods, the duration of the response was clearly that of a 12-hour photoperiod. In the experiment just cited, where the fat and gonadal responses were induced in winter with a 16L–32D schedule, the rate of both responses also exceeded that of a 16L–8D schedule. Hence, a long dark period, instead of being inhibitory, may augment the response to an effective photoperiod. This seems clear from the data available so far in two different experiments, but in view of other variables in the experiments being compared, especially the difference in starting time in the 16L–32D experiment, further testing is necessary and will be undertaken. It is reasonable to conclude from the data reported here that there is a relation between the photoperiod and the dark period, and that an effective photoperiod cannot be negated by a long dark period.

Earlier studies in our laboratory (Wolfson, 1952b) led to the hypothesis that in the progressive phase of the gonadal and fat cycles there are daily physiological increments of response which are a function of effective daily photoperiods, and that the rate of response is determined by the summation of these daily increments. Farner, Mewaldt and Irving (1953) postulated a carry-over effect of the photoperiod into the dark period to explain the effectiveness of short periods of light. They also stipulated the summation of the total daily photoperiodic effect. On the other hand, Kirkpatrick and Leopold (1952) and Jenner and Engels (1952) assigned an active regulatory role to the dark period. All of these interpretations have been derived indirectly from the manifested response of the "effectors"—the gonads and the fat depots. Very little is known about the dynamics of the specific events which regulate the gonadal and fat cycles, but two organs are clearly involved, the hypothalamus and the pituitary (see Wolfson, 1959b, and Farner, 1959, for review and summary). The results of the present experiments lend support to the primary role of the photoperiod, and there is no evidence for an inhibitory role of darkness. When there is no manifest response in a particular photoperiodic schedule, for example 8L–16D, the failure appears to be caused by an ineffective photoperiod, for when the photoperiod was increased to 12 hours a response occurred, even though the dark period remained the same. The difference in rate of response in the 12L–12D and 12L–16D groups suggests that the daily response may be a function of the photoperiod and the subsequent dark period. The dark period could function as a period of time which permits the effect of the photoperiod to continue, as postulated in the carry-over effect, but this would not explain the difference in rate of response in the 12L–12D and 12L–16D groups if the duration of the carry-over effect is the same for a given photoperiod.

One of the questions which was asked in previous studies (Wolfson, 1959a) was whether the bird could "store" the effects of stimulatory light-dark cycles when nonstimulatory cycles intervened. Cycles of 8L–16D were alternated with 16L–8D cycles and the birds responded, but it was not possible to answer the question of storage because the birds could have been responding not to the different daily cycles but rather to the schedule as a whole, for example, 16L–16D–8L–8D, and from other studies it was known that 16L–16D and 8L–8D are stimulatory cycles. The question of storage is perhaps answered by the results of the present experiments. In the 16L–32D cycle, to use an extreme example, the effect of the light period was not negated by the extended dark period. Or, if the bird has a 24-hour rhythm and responded to the 16L–32D cycle on that basis, then it would have experienced alternating days of 16L–8D and 24D. In any case, if gonadotropins are released during effective photoperiods, the testes appear to respond to them each day, or in each cycle, and long intervening dark periods do not inhibit this reaction. Hence, the effects of stimulatory photoperiods appear to be stored each day and summated to the point where the gonadal and fat responses are manifested.

SUMMARY

1. To determine the roles of light and darkness in the photoperiodic responses of birds, effective photoperiods were combined with normally inhibitory dark periods to give cycles longer than 24 hours as follows: 12L–16D; 12L–20D; 16L–16D; 16L–22D; 16L–32D. The experiments were performed in winter and spring, and tested both induction and maintenance of the gonadal and fat responses.

2. The results indicate that the photoperiod and not the dark period determined the response. Long dark periods *per se,* therefore, are not inhibitory.

3. Comparison of the rate of response in previous experiments with similar photoperiods but different dark periods shows a greater rate of response with longer dark periods. The response to a given light-dark cycle may, therefore, be a function of the photoperiod and the subsequent dark period.

4. The results also indicate that the gonadotropic and lipogenetic effects of each stimulatory light-dark cycle are "stored," or cannot be negated by long intervening dark periods.

5. The relation of these findings to theories of the mechanism of response to light and dark is discussed.

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AN ARGINASE INHIBITOR(S) AND ITS POSSIBLE ROLE IN THE DEVELOPMENTAL DECREASE OF ARGINASE ACTIVITY IN CHICK EMBRYOS[1]

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A number of enzyme activities have been measured in crude homogenates of developing embryos, and in several instances changes in activity have been correlated with morphological events and the time tissues and organs begin to function: *e.g.* cholinesterase in amphibian embryos (Sawyer, 1943). Such correlations suggest that enzyme activities measured in crude homogenates reflect events involved in metabolic differentiation. Consequently, it seems reasonable to believe that studies of mechanisms by which enzyme activities change will also yield information concerning mechanisms involved in differentiation at the biochemical level. With this assumption in mind we began studying arginase activity in developing chick embryos in an attempt to establish the cause of the decrease in activity per unit moist weight which occurs during development (Needham and Brachet, 1935; Fisher and Eakin, 1957; Clark and Fischer, 1957; Roeder, 1957).

At the outset of these studies we assumed that the arginase activity expressed by crude homogenates is primarily a measure of the amount of enzyme present. Evidence obtained in the course of surveying activity in various tissues led us to question this assumption. Subsequently, we observed that several tissue homogenates which exhibit little or no arginase activity are capable of inhibiting the activity of embryo homogenates. Preliminary studies indicate that inhibition is due to the presence of a high molecular weight inhibitor(s) and are consistent with the possibility that it is ribonucleic acid in nature. The presence of an arginase inhibitor(s) in chick tissues raises the possibility that the developmental decrease in activity could be due to an increase in the concentration of this agent as well as a decrease in the concentration of enzyme.

MATERIALS AND METHODS

Eggs from a flock of Rhode Island Reds were incubated at 39° C. in a commercial incubator. Eggs were removed at various times, cracked in a pan of water and the entire living area excised. The vitelline membrane was removed and embryo, pellucida, vasculosa and vitellina were separated. At the 24-hour stage no vascular area is present; however, a pre-vascular area exists, containing mesodermal tissue (Hamilton, 1952) which we refer to as vascular area. At several ages, area vitellina and area vasculosa were separated into two layers: ectoderm and endoderm in the case of vitellina, somatopleure and splanchnopleure in the case of vasculosa. The various tissues were weighed and homogenized in distilled

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water in the cold with a pestle homogenizer having a teflon pestle and a glass vessel. Homogenates were assayed for arginase activity using the procedure described below.

Enzyme was activated by heating 4 ml. of homogenate with 0.2 ml. of a 20 per cent solution of $MnCl_2 \cdot 4H_2O$ for 20 minutes at 57° C. Activated homogenate was equilibrated to 30° C. in a water bath and 1 ml. of an 18 per cent solution of arginine hydrochloride at pH 9.5 was added. A saturated solution of NaOH was used to adjust the pH of the arginine solution. One-ml. samples of reaction mixture were removed at 0, 20, 40 and 60 minutes after arginine addition. The samples were heated to 90° C. for 10 minutes to stop enzymatic activity. Two and one half ml. of 0.2 *M* phosphate buffer at pH 6.8 were added to each sample and 0.2 ml. of a glycerol urease preparation from jack bean meal (Folin and Wu, 1919) was used to hydrolyze urea present. After incubation with urease for 10 minutes at room temperature, 2 ml. of half-saturated Na_2CO_3 were added and the ammonia transferred to 0.05 N H_2SO_4 by a stream of ammonia-free air. Nessler's reagent (Folin and Wu, 1919) was added to the acid solutions and the color read on a Klett-Summerson colorimeter, using filter number 54. Ammonia nitrogen present was calculated from a standard curve prepared with analytical grade ammonium sulfate. Nitrogen values for the sample inactivated immediately after arginine addition were subtracted from all values and the corrected values were plotted against time of incubation with arginine. The slope of the resulting curve was used as a measure of arginase activity. In this report activities are presented as μg. arginine hydrolyzed per hour per ml. reaction mixture or per mg. tissue.

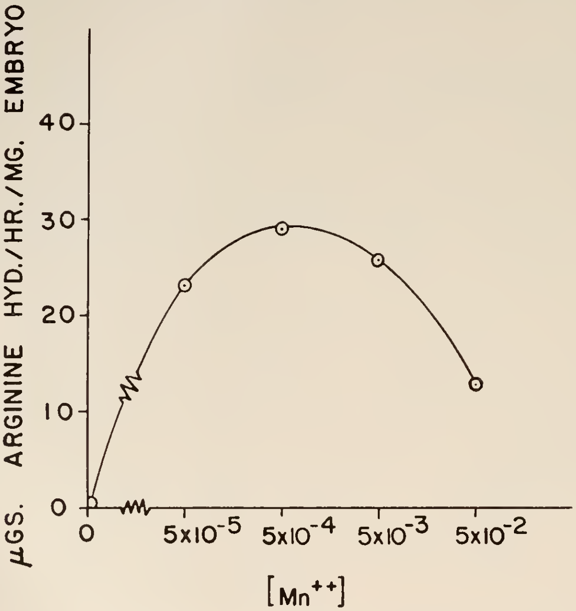

FIGURE 1. Effect of Mn^{++} concentration on arginase activity of 4-day embryo homogenate (15 mg. per ml. reaction mixture).

Activation of arginase

The procedure outlined above gives a 5×10^{-2} *M* concentration of Mn^{++} in the activation mixture. Van Slyke and Archibald (1946) and Greenberg (1955) recommend this concentration to activate liver arginase. We have found that this concentration gives full activity for some but not all chick embryo tissues, *e.g.* 4-day embryo homogenates give maximum activity with 5×10^{-4} *M* Mn^{++} (Fig. 1). In surveying activity changes during development and in experiments involving tissue mixtures, it seemed desirable to use the same concentration throughout. Consequently, 5×10^{-2} *M* Mn^{++} was almost always used. In some experiments involving 4-day embryos 5×10^{-4} *M* Mn^{++} was used. These cases will be noted in the text. Also, $MnSO_4$ in maleate buffer (Greenberg, 1955) was used to activate and found to give the same result as $MnCl_2$ in distilled water.

Van Slyke and Archibald (1946) recommend heating to 57° C. for 20 minutes in the presence of Mn^{++} to activate arginase in crude liver homogenates. Arginase appears to be stable under these conditions. We have found that partially purified liver arginase gives the same activity whether heated or not. Greenberg (1955) recommends heating to 60° C. for 20 minutes as one step in arginase isolation. It should be noted that heating in the absence of Mn^{++} destroys all activity of homogenates (Fig. 1).

Incubation with substrate

The procedure outlined above gives a final arginine concentration of 0.17 *M* in the reaction mixture. A study has been made of 4-day embryo arginase activity at various substrate concentrations. These results give an apparent K_m of 8×10^{-3} *M* which is in good agreement with values of 11.6 and 7.7×10^{-3} reported for liver arginase (Greenberg, 1951).

Most tissues have been assayed at two or more concentrations and in all cases activity appears to be linear with concentration (Figure 5 contains data for 4-day embryo activated at 5×10^{-4} *M* Mn^{++}).

The pH of reaction mixtures prepared as described above is 9.2. Activities have been measured at several hydrogen ion concentrations and maximum activity was found at pH 10, which is in agreement with results reported by Greenberg (1951).

Measurement of urea formed

Phosphate buffer addition to heated samples as described previously gives a pH of 7.5. Urea present has been shown to be quantitatively hydrolyzed by treatment with urease under these conditions. Added urea can be recovered quantitatively from reaction mixtures as ammonia (± 2 μg. N per ml. reaction mixture). When urease is omitted from the procedure, no arginase activity is observed. Arginase activities can be duplicated on the same homogenate, ± 2 μgs. urea nitrogen or ± 12 μg. arginine hydrolyzed per hour per ml. reaction mixture. When no arginase activity was observed, a value was calculated consistent with the sensitivity of the assay (12 μg. arginine hydrolyzed per hour per ml. reaction mixture).

RESULTS

Distribution of arginase activity in developing eggs

The first objective of this study was to describe the changes in arginase activity of several tissues in eggs during the first 7 days of development. Embryo, pellucida, vasculosa and vitellina were excised, weighed and homogenized. An aliquot was removed for dry weight determination. Before the living area was dissected, the distance from the center to the outer edge of pellucida, vasculosa and vitellina was measured (several measurements were made on each tissue in each egg and the averages recorded). From these studies we could follow changes in the moist weight, dry weight, surface area and arginase activity per unit moist weight, dry

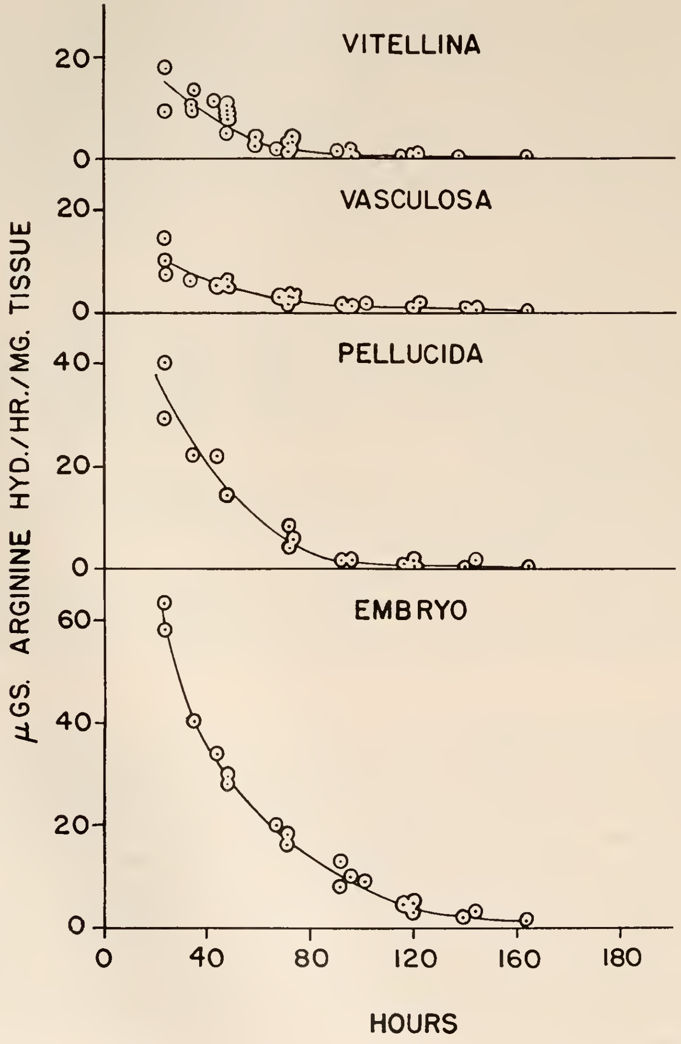

FIGURE 2. Arginase activities of various tissues in developing eggs.

TABLE 1

Arginase activity of tissue layers in yolk sac of developing eggs

Incubation time of eggs—hours	Mg. ectoderm per mg. vitellina	μg. arginine hydrolyzed per hour per mg. tissue			
		Ectoderm	Endoderm	Vitellina	Calculated activity of vitellina
64	0.38	8.2	1.2	1.3	3.9
73	0.37	9.8	2.0	2.6	4.9
65	0.35	13.9	0.0 (<0.9)	1.8	4.8
	Mixture of homogenates—35% ectoderm			1.8	4.8
98	0.33	4.5	0.0 (<2.0)	1.7	1.5
	Mg. somatopleure per mg. vasculosa	Somatopleure	Splanchnopleure	Vasculosa	Calculated activity of vasculosa
73	0.25	8.7	0.0 (<0.5)	1.7	2.2
144	—	1.0	0.0 (<0.3)	0.0 (<0.2)	—

weight and surface area. Embryos increased in moist weight in a pattern almost identical with that reported by Schmalhausen (1926). Pellucida increased in moist weight from 2 mg. at the first day to 300 mg. on the seventh day, vasculosa increased from 10 to 800 mg. and vitellina increased from 46 on the first day to 300 on the third day and subsequently decreased to 180 mg. on the seventh day. Surface areas of pellucida, vasculosa and vitellina followed essentially the same pattern as moist weight, *i.e.*, pellucida and vasculosa increased in area from the first to the seventh day and vitellina increased to a maximum on the third day. An aliquot of each homogenate was dried at 60° C. for two days in a petri dish and the residue weighed. Per cent dry weight was essentially constant in each tissue during the first 7 days of development (embryo 9 per cent, pellucida 10 per cent, vasculosa 17 per cent and vitellina 13 per cent). Arginase activity decreased during development in each tissue when calculated per unit moist weight, dry weight or surface area. Activities calculated per unit moist weight are shown in Figure 2. When activity at the first day was assigned a value of 100 per cent for each tissue, it was found that the pattern of change during incubation was almost the same in all tissues. Area vitellina is of particular interest because of the way it spreads over the surface of yolk. Apparently (Hamilton, 1952), this tissue spreads by addition of cells to its outer edge, which are formed from periblastic syncytium at the zone of junction. If this picture is correct, tissue at the outer edge is younger, relative to the time cells are formed, than in the inner area. We measured arginase activity in a strip of tissue approximately 10 mm. wide around the outer edge of vitellina. From the first to the seventh day this strip exhibits substantially the same activity as the remainder of vitellina. It follows that the mechanisms which cause activity to decrease are operating in the non-cellular syncytium which becomes vitellina; otherwise, activity in the peripheral strip should not decrease as rapidly as in the remainder of vitellina. These observations suggest that all tissues in developing eggs tend to decrease in arginase activity and that the activity decrease is not correlated with any obvious developmental event.

Data in Figure 2 show that tissues exhibit a common tendency to decrease in arginase activity; however, these tissues show marked differences in their level of activity whether calculated on a moist weight or dry weight basis. It appears that there is a gradient of activity related to difference in proximity to yolk. Embryo, the most distant, exhibits the highest arginase activity; pellucida the next most distant, exhibits the next highest activity; and yolk sac tissues in contact with yolk exhibit the lowest activity. Results in Table I show a very significant difference in activity between ectoderm and endoderm of vitellina, and between somatopleure and splanchnopleure of vasculosa. In every case the tissue in contact with yolk material exhibited a much lower activity than the tissue distant from yolk. Consequently, the gradient can be extended to the tissue layers of yolk sac. Furthermore, it was possible to calculate the activity of total tissue (vitellina or vasculosa) from the weights and activities of its component layers (Table I). It was found that at approximately the third day of incubation there was a significant difference between the calculated activity of vitellina and the measured activity. In one case enough of the tissues were obtained so that homogenates of ectoderm and endoderm could be assayed together and separately. In this case endoderm homogenate clearly inhibited the activity of ectoderm. These results are consistent with the possibility that endoderm, at least at one stage, is capable of inhibiting the arginase activity of ectoderm.

Inhibition of embryo arginase activity

Results described in the preceding section suggested that endoderm of area vitellina contains an arginase inhibitor(s). Greenberg (1951) has reported that most amino acids inhibit arginase. It was conceivable that yolk sac tissues contain relatively large amounts of free amino acids from digestion of yolk proteins. An attempt was made to demonstrate inhibitor activity in endoderm using embryo homogenates as sources of arginase activity (Table II). Endoderm homogenates were dialyzed in an effort to remove possible low molecular weight inhibitors (Table II). Dialysis was carried out in the cold against distilled water. Results show that endoderm homogenates do inhibit but that dialysis does not remove inhibitory activity as would be expected if the agents were amino acids. Furthermore, casein hydrolysate, obtained commercially, does not inhibit at a dry weight concentration equal to that of endoderm which is inhibitory. From these results we concluded that inhibition of embryo arginase by endoderm homogenates is not due to free amino acids in the endoderm. It should be noted that dialysis actually increases the inhibitory activity of endoderm homogenates. An explanation of this effect will be presented in a later section of this report.

Preliminary studies of the nature of the inhibitor(s) have been undertaken, using vitellina of eggs incubated 4 days as a source of inhibitor. The dialysis effect described above has been confirmed with vitellina. An attempt was made to destroy inhibitory activity by heating to 90° C. for 20 minutes. Results obtained show that this treatment does not destroy inhibitory activity but actually increases inhibition. These results suggest that the inhibitor(s) is a high molecular weight, heat-stable substance. Moss (1952) reported that sodium ribonucleate from yeast inhibits arginase. We have confirmed this observation, using 4-day embryo as a source of arginase activity. The first two steps of a ribonucleic acid

(RNA) isolation procedure were applied to area vitellina homogenate (Kay and Dounce, 1953). Inhibitory activity was found in the fraction which, in the case of mammalian liver, contains RNA. Four-day vitellina was homogenized in a solution of NaCl and sodium citrate. The homogenate was centrifuged, yielding a supernatant and precipitate (ppt. 1). The supernatant was adjusted to pH 4.5 with HCl and centrifuged, yielding ppt. 2 and supernatant which was adjusted to pH 7.0 with NaOH for testing (final supernatant). The two precipitates were suspended in distilled water. These three fractions were assayed for arginase activity and arginase inhibitor activity using 4-day embryo homogenate as a source of arginase activity. Only ppt. 1 exhibits arginase activity and only ppt. 2 inhibits embryo arginase. Mixtures of ppt. 1 and embryo in three assays yielded higher activity than predicted. This result can be explained by the presence of an arginase stimulatory agent in embryo. Evidence for the existence of such an agent will be presented in a later report.

TABLE II

Inhibition of embryo arginase by endoderm homogenates

Incubation time of eggs—hours	Mg. per ml. reaction mixture		μg. arginine hydrolyzed per hour per mg. embryo
	Embryo	Endoderm*	
70	5.4	0	14
	5.4	2	10
	5.4	3	8.0
	5.4	2 (dial. 1 day)	0 (<2)
	5.4	3 (dial. 1 day)	0 (<2)
96	15	0	7.9
	15	140	4.6
	15	140 (dial. 1 day)	0.8
	15	140 (dial. 4 days)	0 (<0.8)
	15	140 (dial. 7 days)	0 (<0.8)
140	40	0	3.9
	40	9	3.4
	40	12	2.8
	40	9 (dial. 1 day)	1.6
	40	12 (dial. 1 day)	1.2
161	175	0	0.90
	175	125	0.50

* Endoderm homogenates did not exhibit arginase activity alone at the concentrations used in any of these experiments.

These preliminary studies indicate that the inhibitor(s) is high molecular weight in nature and are consistent with the possibility that it is RNA.

Distribution of inhibitor

The presence of inhibitor(s) in tissues which do not exhibit arginase activity can be demonstrated by showing that homogenates inhibit the arginase activity of

4-day embryos, as was done with area vitellina and area vitellina endoderm. It was found that heart from 21-day embryos does not exhibit arginase activity and inhibits 4-day embryo arginase (Fig. 3). As in the case of vitellina and vitellina endoderm, dialysis for 4 days in the cold against distilled water increases the inhibitory activity of heart homogenate (Fig. 3). Heart from 6-week-old chickens also inhibits 4-day embryo arginase. Results obtained show this inhibition when enzyme is activated with 5 × 10^{-2} or 5 × 10^{-4} M Mn^{++}.

In order to directly demonstrate the presence of inhibitor(s) in a tissue which exhibits arginase activity, it is necessary to destroy its arginase activity before testing against 4-day embryo arginase. It is known that dialysis inactivates arginase (Greenberg, 1951). We have confirmed this observation, using partially purified beef liver arginase which was obtained commercially. Activation with Mn^{++} under the conditions we are using does not restore activity. We have destroyed the arginase activity of several tissue homogenates in this manner and tested the dialyzed homogenates for inhibitor activity, using 4-day embryos as a source of arginase activity. It was observed that homogenates of bone marrow, skin, leg muscle, eye and brain from 21-day embryos can completely inhibit the arginase activity of 4-day embryos. The effect of dialyzed brain homogenate is shown in Figure 4. These results clearly show that all tissues tested contain arginase inhibitor(s), if the only effect of dialysis is to inactivate arginase. The observation was made with vitellina, vitellina endoderm and heart that dialysis

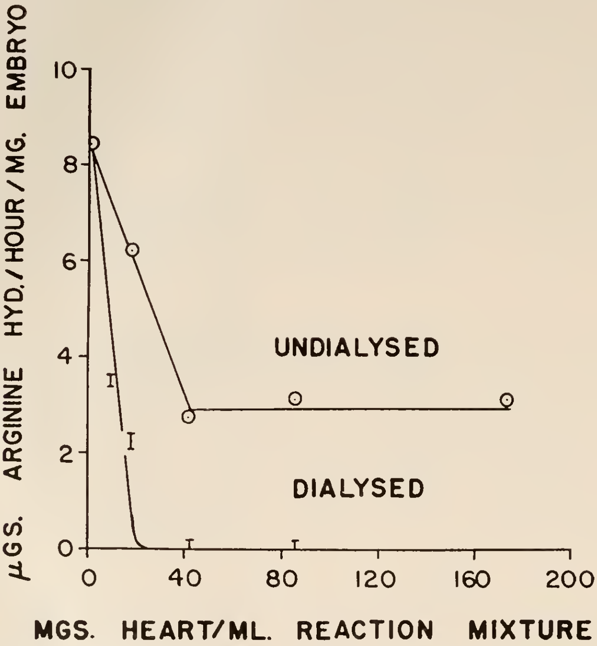

FIGURE 3. Effect of 21-day embryo heart homogenate on the arginase activity of 4-day embryo (15 mg. per ml. reaction mixture). Dialysis was carried out for 4 days in the cold against distilled water.

increases the inhibitor activity of tissue homogenates which express little or no arginase activity. This is inconsistent with the above interpretation unless these tissues do contain arginase which does not exhibit activity because of the presence of inhibitor. Evidence is presented in the following section that heart does contain arginase even though crude homogenates do not exhibit arginase activity. Earlier it was shown that heating vitellina homogenate to 90° C. for 20 minutes, which inactivates arginase, also increases inhibitor activity. These results are consistent with the interpretation that the primary effect of dialysis is to inactivate arginase.

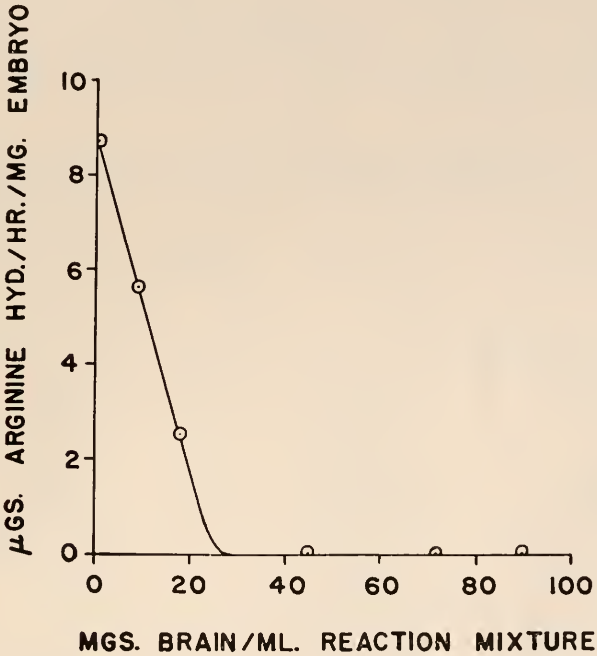

FIGURE 4. Effect of dialyzed brain (from 21-day embryos) homogenate on the arginase activity of 4-day embryo (15 mg. per ml. reaction mixture). Dialysis was carried out for 4 days in the cold against distilled water.

Role of inhibitor in establishing the arginase activity of crude homogenates

If inhibitor(s) is playing a major role in establishing the arginase activity expressed by homogenates, it is necessary that inhibitor(s) be effective against enzyme in the same tissue as well as against 4-day embryo arginase. Homogenate of heart from 6-week-old chickens has been shown to inhibit 4-day embryo arginase. It was found that 8-day embryo heart exhibits arginase activity and that heart from 6-week-old chickens inhibits this activity. A second type of evidence is that dialyzed 4-day embryo homogenate inhibits the arginase activity of undialyzed 4-day embryo homogenate. From these results we believe it is reasonable to assume that inhibitor(s) is effective against enzyme in the same tissue as well as 4-day embryo arginase.

If inhibitor(s) is playing a major role in establishing the activity of crude homogenates, it is necessary that tissues containing inhibitor(s) contain more enzyme than is indicated by the activity of crude homogenates. We attempted to test this possibility by partially isolating arginase from heart of 6-week-old chickens, which does not exhibit arginase activity as a crude homogenate. The arginase isolation procedure recommended by Greenberg (1955) for horse liver was applied to heart (Table III). These results show that heart contains at least 30-fold more enzyme than is indicated by the activity of crude homogenate. It appears that acetone precipitation "unmasks" arginase activity, presumably by inactivating inhibitor since the acetone-soluble fraction does not inhibit 4-day embryo arginase. A similar case has been reported with a pantothenic acid-requiring mutant of Neurospora (Wagner and Guirard, 1948; Wagner, 1949).

Table III

Partial isolation of arginase from hearts of 6-week-old chickens

Isolation step	Fraction	Per cent original N	μg. arginine hydrolyzed per hour per	
			Mg. original tissue	Mg. N in fraction
Heart homogenate incubated overnight with Mn^{++} at pH 7.6	extract A	100	0 (<0.02)	0 (<3)
Acetone precipitation	soluble	6	0.03	81
	insoluble	84	0.62	118
Extraction of acetone Powder with buffer	insoluble	66	0.15	37
	extract B	11	0.26	370
Extract B heated at 60° C. for 20 minutes	precipitate	3	0 (<0.01)	0 (<31)
	extract C	5	0.24	810
Extract C dialyzed and lyophilized	powder D	1.4	0.19	2180

Non-growing pads and homogenates of wild type are capable of coupling pantoyl lactone and beta-alanine to form pantothenic acid. Such preparations of the mutant do not show this activity; however, acetone powders of both organisms can catalyze the reaction. As in the case of arginase, acetone precipitation "unmasks" an enzyme activity.

Possible role of inhibitor(s) in the developmental decrease of arginase activity

Evidence has been presented in a previous section that area vitellina contains an arginase inhibitor(s) and that arginase activity in this tissue decreases during development in a manner similar to other tissues, *e.g.* embryo. Inhibitor activity has been demonstrated in all tissues tested. Results presented in the previous section indicate that inhibitor(s) is playing a role in establishing the activity of crude homogenates. Consequently, the developmental decrease of activity could be the result of an increase in the concentration of inhibitor(s), a decrease in the concentration of enzyme, or the concentration of both agents could change, the net result being a decrease in arginase activity. Even if the concentration of inhibitor remains constant, its presence would cause activity to decrease more than would be warranted by a decrease in enzyme concentration, since there would be an increase in the inhibitor(s)-to-enzyme ratio. The developmental decrease in arginase activity would accurately represent a decrease in enzyme concentration only in the event that enzyme exhibits a *constant* fraction of its activity in various tissues during the developmental period studied. If the modifying influence of homogenates on the activity of arginase is constant in various tissues, mixtures of tissues should exhibit an activity equal to the sum of the activities of the tissues measured separately. Homogenates of several tissues from 21-day embryos were mixed with homogenate of 4-day embryo and the mixtures assayed for arginase activity (Table IV). From these results we concluded that mixtures of tissue homogenates do not necessarily exhibit an activity equal to the sum of the activities of the tissues measured separately. Only in the case of skin was activity equal to the calculated value. Apparently, embryo does not exhibit any activity in the presence of brain or leg muscle. These results are not consistent with the possibility that the modifying influence of all homogenates is the same. Consequently, we believe that the different arginase activities expressed by various tissue homogenates can not be explained simply by differences in enzyme content.

TABLE IV

Arginase activity of mixtures of 21-day embryo tissues with 4-day embryo

Tissue	Mg. per ml. reaction mixture		μg. arginine hydrolyzed per hour per		
			Ml. reaction mixture		Mg. tissue
	Tissue	Embryo	Experimental	Calculated	
Brain	0	20	180		9.0
	90	20	118	298	
	90	0	118		1.3
Leg muscle	0	10	81		8.1
	82	10	69	150	
	82	0	69		0.84
Eye	0	20	149		7.5
	45	20	193	286	
	45	0	137		3.0
Bone marrow	0	10	69		6.9
	36	10	69	88	
	36	0	19		0.53
Skin	0	10	56		5.6
	63	10	124	125	
	63	0	69		1.1

Results described in Table IV can be explained with the assumption that tissue homogenates are effectively mixtures of enzyme and inhibitor(s), provided the enzyme-inhibitor interaction is reversible. Evidence for reversibility has been obtained, using 4-day embryo as a source of arginase activity and heart from 6-week-old chickens to inhibit. Results presented in Figure 5 show that this system exhibits the properties which, according to Ackermann and Potter (1949), are characteristic of reversible inhibition. If tissue homogenates are effectively mixtures of enzyme and a reversible inhibitor(s), mixtures should exhibit activities equal to or less than the sum of the components but equal to or greater than the activities of the least active components. Results in Table IV are consistent with these requirements. If the activity of a tissue homogenate is determined by the presence of a reversible inhibitor as well as enzyme, it is possible that activity would not be proportional to tissue concentration. Data in Figure 5 show that the arginase activity of 4-day embryos is proportional to tissue concentration over a 10-fold range. Such a proportionality has been shown by Straus and Goldstein (1943) when the ratio of inhibitor concentration to dissociation constant is greater than 100 (zone c). Our results can be explained on the basis of zone c inhibition.

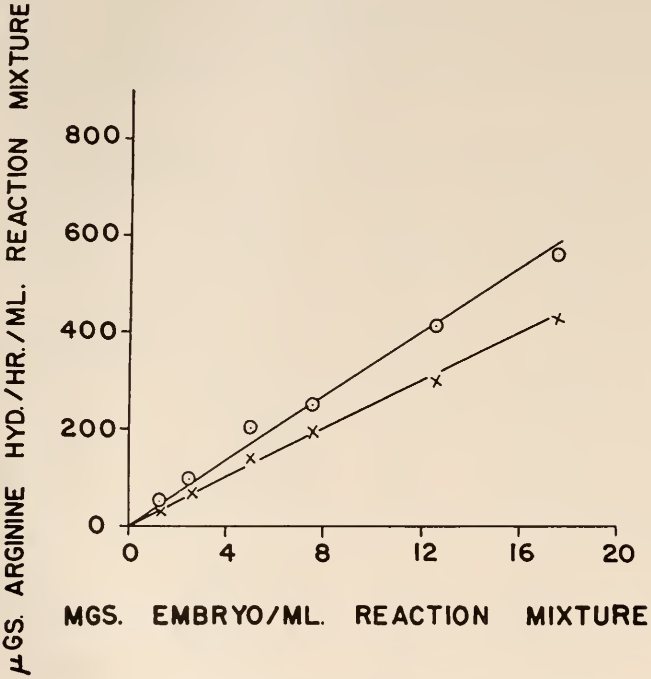

FIGURE 5. Effect of heart from 6-week-old chickens (120 mg. per ml. reaction mixture) on the arginase activity of 4-day embryo at various concentrations. Activated with 5×10^{-4} *M* Mn^{++}. ⊙ embryo alone, X plus heart.

Assuming that homogenates are effectively mixtures of enzyme and inhibitor(s), the data in Table IV require that different tissues have different inhibitor(s)-

to-enzyme ratios. Consequently, enzyme does not exhibit the same fractional activity in all tissues and, therefore, inhibitor(s) must be playing some role in the differentiation process which results in each tissue having its characteristic arginase activity, as measured in homogenates. This suggests that the developmental decrease in activity could also be at least in part due to a change in inhibitor concentration. We have found that heart arginase activity decreases between the eighth and the twenty-first day of incubation. At the twelfth day, heart exhibits a small but significant arginase activity and does not inhibit 4-day embryo arginase. At the twenty-first day, heart does not exhibit a measurable arginase activity and inhibits 4-day embryo arginase (Fig. 6). These results show that a decrease in arginase activity is correlated with an increase in the ability to inhibit embryo arginase.

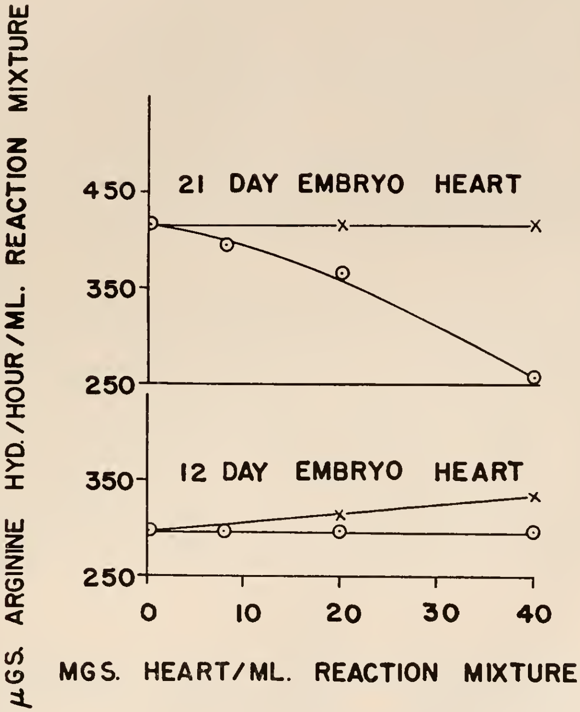

FIGURE 6. Effect of heart from 12- and 21-day embryos on the arginase activity of 4-day embryos (12.5 mg. per ml. reaction mixture) activated with 5×10^{-4} *M* Mn^{++}. ⊙ embryo-heart mixture, X embryo activity + heart activity as measured separately.

Evidence presented in this section suggests that the arginase activity expressed by tissue homogenates is determined by the concentrations of both enzyme and inhibitor(s), different tissues contain different relative amounts of inhibitor to enzyme and that in one case the developmental decrease of arginase activity is correlated with an increase in inhibitory activity. We believe these results demonstrate that inhibitor(s) plays some role in the developmental change in arginase activity.

DISCUSSION

Results reported here suggest that ribonucleic acids could be inhibiting arginase activity in crude chick embryo homogenates. Sorm and Hrubesova (1955) found that ribonucleic acid inhibits chymotrypsin and trypsin; Moss (1952) found that ribonucleic acids inhibit arginase; Klingenberg (1952) reported that cathepsin is inhibited by ribonucleic acid; Chepinoga and Pavlovskii (1956) found that ribonucleic acid and deoxyribonucleic acid inhibit aldolase and enolase; and Zittle (1946) reported that succinic dehydrogenase is inhibited by both deoxyribonucleic acid and ribonucleic acid. A large number of cases have been reported where proteins inhibit enzyme activities (*e.g.* Swartz, Kaplan and Frech, 1956). These numerous instances where high molecular weight inhibitors have been found in various tissues and organisms raise the possibility that enzyme activities of crude homogenates are not necessarily a measure of enzyme content.

Changes in the enzymic activity of developing embryos, as measured in crude homogenates, have been demonstrated in numerous instances and it seems reasonable to believe that these changes constitute one major aspect of differentiation. It would be of considerable interest to identify the mechanisms that are responsible for these observed changes. Before we can understand such mechanisms, we must establish the meaning of the change in activity. It is conceivable that an enzyme activity, as measured in crude homogenates, could change during development in at least three ways: (1) in the amount of enzyme per unit tissue, (2) in the nature of the enzyme, and (3) in the modifying influence of substances present in the homogenate. Markert (1958) has obtained evidence that embryos produce different esterases at different stages of development. Results presented in this report suggest that in the case of arginase, substances which modify its activity may play a role in the developmental change. Consequently, it is clear that the nature of the change which is expressed as a change of enzymic activity must be established before an attempt can be made to determine the mechanism causing the change.

SUMMARY

1. Arginase activity was measured in tissues of developing eggs from the first to the seventh day of incubation. Activity per unit moist weight or dry weight decreased in embryo, pellucida, vasculosa and vitellina.

2. Evidence was obtained that all chick tissues studied contain a high molecular weight arginase inhibitor(s) which appears to be ribonucleic acid in nature.

3. Inhibitor(s) seems to play a major role in establishing the level of arginase activity expressed by crude homogenates.

4. The decreased arginase activity of hearts from developing embryos has been correlated with an increase in inhibitor activity.

5. These results suggest that the developmental decrease in arginase activity could be the result of an increase in inhibitor concentration as well as of a decrease in enzyme concentration.

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